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The Contribution of Tetraploid Cells to Mid-Gestation Mouse Aggregation Chimaeras

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DECLARATION

Except where due acknowledgement is made, the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Roberta Margaret James

March 1993

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ABSTRACT OF THESIS (Regulation 3.5.10)

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Title of Thesis The contribution of tetraploid cells to mid-gestation mouse aggregation chimearas

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.Mouse aggregation chimaeras were used in this study to attempt to evaluate the developmental potential of tetraploid cells in the fetus and extraembryonic membranes of mid-gestation chimaeric conceptuses.

Diploid↔diploid chimaeras were generated and analysed to provide a control and general baseline for the analysis of tetraploid↔diploid chimaeras. Relationships between compositions of tissues in the diploid↔diploid chimaeras were in agreement with those already established.

Tetraploid embryos were produced by electrofusion, which facilitates the fusion of adjacent blastomeres following the exposure to electric field pulses.

Three genetic cell markers were chosen to allow identification of the two cell populations in chimaeric conceptuses. These were; (i) pigment in the retinal epithelial cells of the eye; (ii) electrophoretic variants of the enzyme glucose phosphate isomerase (GPI); (iii) the ß-globin transgenic marker, which can be detected by *in situ* hybridization.

The bulk of the analysis was performed at $12^1/_2$ days of gestation by assaying homogenized tissue for GPI. By constructing chimaeras from two embryos which were homozygous for different alleles of *Gpi-1*, it was possible to estimate the proportions of each cell type in each tissue of the chimaera. *In situ* hybridization was used to provide spatial information on the yolk sacs of the tetraploid↔diploid chimaeras. The efficiency of the electrofusion technique was tested by *in situ* hybridization, to ensure that embryos produced by the technique were uniformly tetraploid and not 4n/2n mosaics.

In the tetraploid↔diploid chimaeras, the tetraploid cells contributed mainly to the primitive endoderm derivatives (yolk sac endoderm, parietal; endoderm), with very little contribution to the primitive ectoderm derivatives (fetus, amnion, yolk sac mesoderm). There was also good colonisation of the trophectoderm derivatives (trophoblast, placenta).

Since the cells of tetraploid embryo in a tetraploid↔diploid chimaera are larger than the diploid cells, the pattern of colonisation of tetraploid cells in chimaeric conceptuses may have been due to the cell size rather than because the cells were chromosomally abnormal. To test this theory, chimaeras were constructed by aggregating a diploid 4-cell embryo with an 8-cell embryo. After analysis, the distribution of cells from the diploid 4-cell embryo in chimaeric conceptuses closely resembled that of tetraploid cells.

Tetraploid cells do not colonise all tissues of mid-gestation mouse chimaeras equally. Some selection of tetraploid cells exists, whether due to altered cell properties, or merely to cell size, which prevents tetraploid cells from colonising the primitive ectoderm derivatives efficiently. There are three possible mechanisms to account for this finding; (i) abnormal cells are usually excluded from the inner cell mass; (ii) abnormal cells are usually excluded from the primitive ectoderm derivatives; (iii) tetraploid cell can contribute to all three lineages early in development, but are selected against in the fetus. There is insufficient data to determine which of these mechanisms is in play, and it is possible that a combination of two or more of these mechanisms exists.

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CHAPTER 1.
INTRODUCTION

The experimental work described in this thesis involves the analysis of the distribution of diploid and tetraploid cells in different developmental lineages of mid-gestation chimaeric mouse conceptuses. This first chapter provides an introductory review of polyploidy, the establishment of the developmental lineages which were analysed in the study and the uses of mouse chimaeras in experimental embryology.

1.1 POLYPLOIDY

The phenomenon of polyploidy is a well documented, natural occurrence in many forms of life. Some 30-45% of flowering plant species have multiples of the chromosome number characteristic of the genus to which they belong (deWet, 1980); at least 70% of monocotyledons have a history of polyploidy in their evolution (Goldblatt, 1980). Polyploid species, such as wheat, rye and sweet potato are important in agriculture as they tend to be larger and have greater adaptability to the environment. The larger size is useful in food production, but also for other commercial reasons, for example, in cut flowers (Zeven, 1980).

The success of polyploidy in plants is not reflected in the animal kingdom. The number of polyploid animals compared to diploid is very small, because of the difficulties encountered by polyploid cells at meiosis, which plants can overcome by vegetative reproduction. From an estimated $2^1/_2$-3 million insect species, less than one hundred have been found to be polyploid (Lokki and Saura, 1980). Those studied, though, are successful, colonizing vast areas, usually northerly and mountainous habitats. The mode of reproduction is always parthenogenetic in polyploid insects. It was thought that the large extent of gene duplication in polyploids possibly nullified

the effect of new mutation, making adaptation an unlikely event. This, along with parthenogenesis, a so called "dead end method of reproduction", since no genetic recombination occurs, has led people to believe that polyploidy has played a very minor role in the evolution of animals (Schultz, 1980).

However, more recently, it has been postulated that polyploidy represents an important mechanism for adaptation, speciation and evolution (Nagl, 1990). The additional genetic material required for fish to evolve to terrestrial tetraploids was probably gained by polyploidization (Schultz, 1980). Polyploid species of fish do occur, for example the tetraploid salmonids. Studies of the linkage relationships between salmonid species (Johnson *et al,* 1987) strongly suggest that all modern salmonids arose from a common ancestral tetraploid progenitor.

There is a marked increase in the DNA content of amphibians, compared to fish. An ever increasing number of polyploid amphibians and reptiles have also been discovered (Bogart, 1980). Polyploid reptiles are female triploids which reproduce parthenogenetically, whereas amphibians have several modes of reproduction. Female polyploid eggs may initiate cleavage spontaneously (parthenogenetic) or may require stimulation from a male of some bisexual species (gynogenetic). Alternatively, hybridogenetic reproduction occurs, where polyploid bisexual males and females contribute equally to the zygote and one of the parental genomes is eliminated from the F_1. From this brief account, it is clear that there are some invertebrates and certain vertebrates, such as fish, amphibians and reptiles, which are able to tolerate and compensate for polyploidy throughout the whole organism.

Mammalian polyploids however, are not viable, and although genetic balance appears to be maintained (Epstein, 1986), are developmentally abnormal if they survive long enough in gestation. It is interesting to note, however, that tetraploid cells are frequently found in amniotic fluid cell cultures to be used for prenatal diagnosis. It is

not unusual for detection to be as high as 50% and to be considered normal, as it is suggested that tetraploid cells occur normally in the amnion (Gardner, 1982). Tetraploid cells have also been observed in cultured epithelium from patients with hereditary tumours (Gardner syndrome; Danes, 1976), haematopoietic disorders, tumours of the female reproductive tract and instances of partial hydatidiform mole (Surti *et al*, 1986), as well as some human cell lines, such as liver, striate cortex, leucocytes and bone marrow (Wilson *et al*, 1988). Annerén (1982), reported an increase from normal to tetraploid cells in skin fibroblast cultures of individuals with reduction malformations due to *in utero* exposure to the drug thalidomide.

POLYPLOIDY IN HUMANS

When compared to other animals, it is surprising to discover that humans are quite inefficient at reproducing. Approximately 65-75% of all implanted embryos may be lost (Valentine, 1986), mainly due to intrinsic defects in the zygote or the developing embryo. The loss is rarely due to maternal factors. Chromosome disorders are responsible for much of the embryo wastage. The majority of spontaneous abortions take place during the first eight weeks of gestation, and it is reported that around 70% of these such embryos have some form of chromosome abnormality (Valentine, 1986).

During the first trimester of pregnancy approximately 50% of all aborted fetuses show evidence of chromosome abnormalities. Beyond 16 weeks of gestation the figure drops to around 10% of all spontaneous abortions. Of the 50% of first trimester spontaneous abortions, only 15% of these can be accounted for by polyploidy. Trisomy is responsible for approximately a third, the most common being trisomy 16. Abnormalities which account for the remainder of spontaneous abortions include; translocations (balanced and unbalanced); monosomies, which are very rarely seen, but must occur to balance out the trisomies; and sex chromosome abnormalities.

The number of liveborn infants with a chromosome abnormality is around one in two hundred (0.5%). The most common abnormality at birth is trisomy 21 or Down's Syndrome. These figures emphasise the impact that chromosome disorders have on human pregnancy, as well as the necessity for understanding the mechanisms which bring about abnormal chromosome complements in the embryo, and in the parental gametes.

Although somewhere in the region of 1.3% of first trimester abortuses are tetraploid (Scarbrough *et al*, 1984), very few cases of liveborn infants have been reported (Golbus *et al*, 1976; Pitt *et al*, 1981; Scarbrough *et al*, 1984; Lafer *et al*, 1988; Shiono *et al*, 1988; Pajares *et al*, 1990). Several theories have been proposed to describe the events which may give rise to a tetraploid conceptus. Firstly there may be trispermic (3 haploid sperm) or dispermic (1 haploid and 1 diploid sperm) fertilization of a haploid ovum (Sheppard *et al*, 1982; Surti *et al,* 1986). Alternatively fertilization by either two haploid or one diploid sperm after suppression of first or second maternal meiotic division (Scarbrough *et al*, 1984) may occur. Sheppard *et al* (1982) reported a tetraploid conceptus which had 1N maternal and 3N paternal contributions, indicating a trispermic origin, but generally the sex chromosome complement of reported tetraploid cases is either XXYY or XXXX. This fact along with information obtained by performing chromosome banding techniques (Kajii and Niikawa, 1977) on abortuses and their parents strongly suggests that tetraploidy generally arises due to suppression of cell division at the first cleavage division of the zygote (Pitt *et al*, 1981).

By far the greatest number of polyploid spontaneous abortions are partial hydatidiform moles; 86% of triploid conceptuses abort as partial moles. Most partial moles are triploid with two paternal sets of chromosomes; a result of dispermy. A smaller proportion are triploid due to non-disjunction at maternal meiosis (Gardner and Sutherland, 1989). Partial molar pregnancies, can be recognised by their characteristic

histology; the presence of fetal tissue, two populations of chorionic villi (those with and those without molar degeneration) and focal trophoblastic hyperplasia, and usually miscarry in the first or second trimester. A few cases of tetraploid and tetraploid/diploid partial mole have been reported. 4n/2n mosaic moles reported by Tóth *et al* (1992) were indistinguishable from their triploid counterparts, whereas purely tetraploid partial moles displayed histology which ranged from hydropic abortuses (characterized by an excess of fluid) to partial moles.

It is thought that a number of tetraploid partial moles arise, as other tetraploid conceptuses, by suppression of the first cell cleavage, and may simply be an empty gestational sac, not displaying the cistern (fluid filled spaces) formation and trophoblastic hyperplasia typical of a triploid partial mole (Surti *et al* , 1986). A few tetraploid moles do exhibit three paternal sets of chromosomes. Surti *et al* (1986) reported on two characteristic partial hydatidiform moles with the karyotype 92, XXXY. It is known that maternal and paternal gametes are not developmentally equivalent (McGrath and Solter, 1984; Surani *et al*, 1984; Cattenach and Kirk, 1985;) and it may be that the ratio of maternal:paternal haploid sets of chromosomes has an important bearing on the resulting phenotype of a zygote.

In all eight of the reported liveborn cases of pure tetraploidy, the infant was born with multiple congenital malformations. The most common manifestations include: microcephaly or small head and small lower jaw (micrognathia); a prominent, narrow forehead with hypertelorism (decreased distance between the eyes); micropthalmia/ anopthalmia (small or absent eyes); short palpebral fissures (eye slits); low set ears with under-developed cartilage; arched or cleft palate; beaked nose; positional and structural limb defects, including arachnodactyly and flexion deformities; genital ambiguity and in males cryptorchidism (failure of the testes to enter the scrotum); meningomyelocele (hernia of the spinal cord); decreased muscle tone ; congenital heart disease; microgyria or abnormally small malformed convolutions of the brain.

Survival of tetraploid infants ranges from 11 hours (Scarbrough *et al*, 1984) to 15 months (Shiono *et al*, 1988; still alive at time of their report).

Of the eight reported liveborn cases, five were male and three were female. It is not possible, from this small group to determine whether the male:female ratio is balanced or not. In a cytogenetic study of tetraploid spontaneous abortions (Sheppard *et al*, 1982), 75 were 92,XXXX and 45 were 92,XXYY. This ratio suggests that XXYY tetraploid conceptuses are less viable than XXXX. Snow (1975) suggested that X-inactivation may play a part in the developmental abnormalities seen in tetraploid mouse embryos. The correct balance is thought to be one active X-chromosome per two sets of autosomes and that an upset in this balance may lead to developmental problems. If this were the case in tetraploid cells, two active and two inactive X-chromosomes in an XXXX genome would result in genetic balance, as would one active and one inactivated X-chromosomes in an XXYY genome. This reasoning is backed up by evidence from mid-gestation tetraploid mouse embryos (O'Neill *et al*, 1990) where the sex ratio was close to the predicted 1 XXXX:1 XXYY. It is not known why there should be a discrepancy between the human data and that of the mouse. It may be that the XXXX human conceptuses can survive longer in development than the XXYY, and if it was possible to study spontaneous abortuses earlier in gestation, a sex ratio of 1:1 may also emerge from the human data.

The causes of tetraploidy are largely unknown. Unlike other chromosome abnormalities, increased parental age appears not to be a factor, since the average maternal age in the eight liveborn cases studied was 24. No consanguinity was reported between the parents of any of the tetraploid infants. One possible causative factor was discussed by Carr (1970), who observed that the frequency of polyploidy in spontaneous abortions increased in pregnancies occurring within 6 months of discontinuing oral contraceptive.

The study compares a group of women who became pregnant, and had spontaneous abortions, whilst taking oral contraceptives or within six weeks of ceasing them, with a large unselected group of women who had spontaneous abortions. The level of the most common anomaly, trisomy, remained constant between the groups, whereas the instances of triploidy was increased by four fold in the contraceptive group. The incidence of tetraploidy was very low in both groups and although the numbers were too small for reliable inference, the instance of tetraploid abortions also increased (roughly six fold). Since the level of trisomy was unchanged between groups, it appears that the polyploid data is a real phenomenon.

Carr (1970) proposed several theories to account for the increase in polyploidy after recent exposure to oral contraceptives, concentrating mainly on triploidy. It is well established that oocyte release is controlled by follicle stimulating hormone (FSH), luteinizing hormone (LH), oestrogen and progesterone. What is not so well known is how soon pituitary hormone levels return to normal, what long term effects exposure to oral contraceptives has on the ovary and how this affects normal oocyte release and transport. Several studies have excluded oestrogen and oestrogen-progesterone combinations from human teratogenesis (Shardein, 1985), and Kajii and Niikawa (1977) failed to detect the correlation between an increase in polyploidy with recent exposure to oral contraceptives, found by Carr, during their studies.

In two of the three cases of liveborn tetraploid infants reported by Scarbrough *et al*, (1984), the mother conceived whilst taking oral contraceptives. Kelly and Rary (1971) reported that the mother of a mosaic tetraploid female had been given "a hormone injection" at two months of gestation "to prevent a pregnancy". In another instance (Wilson *et al* ,1988) the mother conceived while receiving the drug Clomid, which is an anti-oestrogen used to stimulate ovulation in cases of infertility. The evidence of tetraploid/diploid mosaicism in a mother and two daughters with polycystic ovary syndrome (Rojanasakul *et al*, 1985) and polyploid tumours of the reproductive tract

suggest that there may be some connection between polyploidy and reproductive hormones.

Oestrogens have been shown to inhibit mitosis in cultured Chinese hamster cells (Wilson *et al*, 1988), and if there was a failure to extrude the first polar body or if the oocyte cytoplasm failed to cleave in time with the division of the chromosomes, say at meiosis II, triploidy may ensue. Failure to cleave at the first zygotic cleavage may allow tetraploidy to occur. It must be borne in mind that these are not the most common causes of triploidy (Kajii and Niikawa, 1977), dispermy being the most common origin. Singh and Carr (1970) tested the effects of a group of different estrogens, progesterones and gonadotrophins on somatic cell division *in vitro*, using human lymphocyte cultures. They found no increase in polyploid cells after exposure to any of the hormones, which implies that either there was no direct effect on dividing cells, or that their model was not entirely suitable since tissue culture cells are not comparable to germ cells. Human postmenopausal gonadotropin and human chorionic gonadotrophin are known to cause changes in the zona of rhesus monkey oocytes (Carr, 1970), which suggests the possibility of dispermy occurring.

An alternative hypothesis on the origin of polyploidy in human conceptuses has been put forward by Carr (1970) and Kajii and Niikawa (1977), which suggests that delayed ovulation and/or fertilization may lead to dispermy (or trispermy) and ultimately to polyploidy. If fertilization of the mammalian ovum is delayed, the shedding of the second polar body, or the normal blocks to polyspermy may be defective (Carr, 1970). Although demonstrated in animals, it is difficult to prove delayed ovulation or fertilization in humans. Boué *et al*, (1975) studied the length of menstrual cycles in women who had spontaneous abortions with chromosome anomalies, and found 25% of polyploid fetuses among abortuses from women who had an abnormally long menstrual cycle (32 days or more) compared to 16% in women with a normal cycle.

It is interesting to note the similarities between tetraploid infants and those with other chromosomal abnormalities; namely triploidy and trisomy. Survival of triploid conceptuses beyond 22 weeks is rare (Lodeiro, 1989) and only 1 in 10,000 approaches term. There are only 50 or so reported cases which have survived beyond that age. Triploid fetuses show a number of common clinical stigmata and congenital malformations which overlap the profile of tetraploid cases. Some of the features common to both abnormalities include; prenatal growth retardation, defects of the eye (colobomas; gaps in the structure of the eye and micropthalmia), micrognathia, facial clefting, low set malformed ears, syndactyly, genital abnormalities (in the male), congenital heart defect and abnormal formation of the cranial bones and brain. There is a set of further features which do not appear to manifest in tetraploidy, as some characteristics of tetraploidy are not seen in cases of triploidy. The major of those related to triploidy are; an abnormally small amount of amniotic fluid, an abnormally large and/or hydropic placenta, horseshoe kidneys, rocker bottom feet and small adrenal glands.

Of the trisomies, trisomies 13 and 18 have a number of features in common with polyploidy (Valentine, 1986). Infants with trisomy 13 are described as being of low birth weight, and are commonly microcephalic, the fore brain usually the area which is underdeveloped. Other features are micropthalmy/anopthalmy; cleft lip and palate; small ill-formed low set ears; polydactyly; congenital heart defect and genital malformations. Trisomy 18 infants suffer many similar malformations; microcephaly; flat low set ears; beaked nose; congenital heart defect; cryptorchidism; lumbar meningomyelocele; rocker bottom feet and joint flexion. Several questions arise from these observations. Firstly, are the malformations which are common among triploidy and tetraploidy and one of the trisomies described due to duplication of that particular chromosome giving what amounts to a "pseudo-trisomy".

If this were the case surely features common to other trisomies, say Down's syndrome, would be visible in the polyploid phenotype. Because this is not the case perhaps the effects are due more to lack of controlling factors for genes or specific regions on say, chromosome 13 or 18, which gives a common phenotype to the polyploidies and the trisomies. Since tetraploids have a complete duplication of the whole diploid genome, it would seem reasonable to expect that although expression of genes may double, that controlling factors would also be doubled, keeping the organism in genetic balance. Clearly this is not the case.

It may be possible to shed light on the relationship between gene dosage and abnormal chromosome complement by regarding tetrasomies (2 extra copies of a given chromosome or section of a chromosome). Autosomal tetrasomy has been reported for regions in several chromosomes and for all of chromosome 21 (Epstein, 1986). The obvious question is whether tetrasomy results in new abnormalities or more severe abnormalities than the trisomy for the same region does. When abnormalities associated with tetrasomies are compared to those resulting from the corresponding trisomies, the results range from little or no specific phenotype differences to clearly distinguishable syndromes. For instance, tetrasomy for 9p is quite similar to trisomy 9p, although perhaps more severe, while tetrasomy 18p produces a phenotype which differs significantly from the corresponding trisomy. Trisomy 18p produces no specific dysmorphic features and is associated with mental retardation in only some cases; tetrasomy 18p results in a wide range of neurological and physical abnormalities. In the mouse, tetrasomies for small regions may be compatible with viability and may cause an increase in severity of the phenotype, whereas in the case of tetrasomy for the entire chromosome 16, mouse fetuses died earlier and were more severely retarded (Debrot and Epstein, 1986).

The majority of human liveborn tetraploid cases reported have been mosaics with a tetraploid and another cell line, usually 4n/2n (Kelly and Rary, 1974; Veenema *et al*,

1982; Scarbrough *et al*, 1984; Rojanasakul *et al*, 1985; Quiroz *et al*, 1985; Wittwer and Wittwer, 1985; Aughton *et al*, 1988; Wilson *et al*, 1988; Warburton *et al*, 1991; Wullich *et al*, 1991). Mosaicism may arise due to failure of cytoplasmic cleavage at a mitotic division later in development than the first cleavage. In some cases 4n/2n mosaic individuals are only diagnosed after presenting with a well defined clinical syndrome such as Ullrich-Turner or Noohan (Wilson *et al*, 1988), whilst others display a range of features typical of complete tetraploidy.

Using the same logic as the trisomy/tetrasomy argument, it would seem sensible to expect the phenotype of a tetraploid infant to be more severe than a triploid, and for complete polyploids to have a more severe phenotype than their respective mosaics. In some cases, a low percentage of 4n/2n mosaicism is associated with a near normal phenotype (Wilson et *al*, 1988). This type of mosaicism may be the result of errors in cell division much later in development. On the other hand, Veenema *et al* (1982) described an infant with only 16% tetraploid cells in peripheral blood lymphocytes which had a very severe phenotype and died shortly after birth. Aughton *et al* (1988) described an infant with a high percentage of 4n/2n mosaicism (95% tetraploid cells) in the bone marrow, but no tetraploid cells in either the lymphocytes or the skin, which had a phenotype as severe as many full tetraploids. There are several explanations for this unusual finding. The error in cell division may have occurred in only the mesoderm, the tetraploid cells failed to survive in other tissues, or the author failed to culture tissues which had a significant percentage of tetraploid cells. It is clear that the percentage of tetraploid cells in a mosaic individual would depend on the point in development when the error occurred; the later the event, the narrower the band of affected tissues. In a case described by Quiroz *et al* (1985), the percentage of tetraploid cells in the peripheral blood of an infant decreased from 68% to 12% in just six months. This finding strongly suggests that cell selection against the abnormal cell population was occurring, and may have increased the individual's survival.

With respect to triploid conceptuses, many are aborted and found as severely retarded, but well formed fetuses. On the other hand the majority of tetraploid abortuses are empty gestational sacs. However, evidence suggests that the phenotype of liveborn triploids is usually more severe than tetraploids, as the majority die within a few weeks of birth. It is also true that tetraploids and triploids differ in their sex chromosome constitution. Technically triploids have a sex chromosome aneuploidy, whether one or two X-chromosomes are inactivated. It has been suggested that the severity of the triploid phenotype may differ according to whether one or two X-chromosomes are inactivated or whether there are two maternally derived and one paternally derived chromosome present, or vice versa (Epstein, 1986). A similar effect may be possible in tetraploids, dependant on how many X-chromosomes are inactivated, and perhaps the ratio of maternal:paternal inactivated.

Larger cells are seen in polyploid mosaics, and it has been suggested by Epstein (1986) that critical ratios of genome/nucleus/cytoplasm may be altered in polyploids. If it is assumed that the cellular volume of triploids is 50% greater than diploid cells, and tetraploids twice as great, and the cell surface:volume ratio remains constant, it is obvious that the cell must assume a different shape. It is possible that a cell would synthesize the correct area of cell membrane, if the gene dosage rate was normal. The other alternative is for a reduction in the cell surface:volume ratio. Either of these alternatives would lead to aberrant development, since it can be assumed that larger cells, abnormally shaped cells or cells with an altered cell surface will be unable to interact normally in a dividing, developing organism. If only these geometric conditions applyed then the consequences of triploidy should once again be less severe than tetraploidy, which does not seem to be the case.

It is obvious from the case histories cited that polyploidy in humans is a complex syndrome with many interacting causative factors, such as the problems of cell size and cell interactions, gene dosage and altered autosome:sex chromosome as well as X-

inactivation. No environmental factors have been shown irrefutably to cause polyploidy, but by studying the origins and mechanism by which polyploidy arises it may be possible in the future to identify putative agents.

EXPERIMENTALLY INDUCED TETRAPLOIDY IN ANIMALS

Many methods have been devised to induce tetraploidy experimentally in mammalian embryos, in order to develop a suitable animal model for the study of the behaviour of polyploid cells within the embryo as a whole, and to gain insights into the phenomenon of polyploidy in the human context.

As early as 1939, Pincus and Waddington attempted to suppress cleavage of rabbit ova using several treatments; ether, ethyl alcohol, colchicine and exposure to elevated temperature. All of the methods suppressed cleavage to a degree, but they found colchicine gave the best results, producing tetraploid ova. Other groups have utilized the technique of suppressing the first cleavage division of fertilized eggs using colchicine (Edwards, 1958) or cytochalasin B. Edwards injected colchicine through the cervix of female mice into the uterus, before mating them to male mice. About 10% of embryos recovered at $3^1/_2$ days of gestation were heteroploid, mostly triploid. No heteroploids survived to maturity and high concentrations of colchicine adversely affected development of the embryo.

Attempts to suppress the second cell cleavage with cytochalasin B have proved more successful for Snow (1973, 1975, 1976), Tarkowski *et al* (1977) and Lu and Markert (1980). Cytochalasin B, which disrupts microfilaments, suppresses cytokinesis without affecting karyokinesis. Snow (1975) reported that only a small proportion (17%) of treated mouse embryos were capable of postimplantation development, but development could advance to term. Many tetraploids at the blastocyst stage lacked an inner cell mass (ICM) and appeared as trophoblastic vesicles. Postimplantation tetraploid embryos were small compared to diploid ones of a similar stage, and the

fetal, but not placental weight was reduced. Up to $13^1/_2$ days embryonic development appeared normal, but after that point in gestation, many developmental aberrations were found. The most common general defect was the presence of haemorrhage and interstitial blood. The yolk sac derived blood, the gonads, the eyes, the kidneys, the anterior end of the neural tube and the brain were frequently abnormal.

Tarkowski *et al* (1977) did not obtain mouse embryos at such advanced stages of development. In their hands tetraploid embryos showed a definite developmental anomaly which manifest at about 8 days of gestation. After 8 days, the development of the embryonic part of the egg cylinder was retarded, and overall growth slowed. The yolk sac and ectoplacental cone seemed to form reasonably well, as did amnion and allantois in some instances, but the embryonic region never amounted to more than a rudimentary hind region of the embryo and was mostly either absent or a disorganised clump of cells. It is obvious that the findings of Snow and Tarkowski *et al* are significantly different, most probably due to different genetic backgrounds, as discussed more fully in Chapter 3.

Another method of producing tetraploid embryos experimentally is by microsurgical transfer of nuclei from morula cells to fertilized one cell eggs (Modlinski, 1978). More than 20% of manipulated mouse eggs developed to the morula or blastocyst stage as tetraploids. The third major way of producing tetraploids is by blastomere fusion. Previously the production of mouse tetraploids by cell fusion involved either virally-assisted blastomere fusion employing inactivated Sendai virus (Graham, 1971; O'Neill *et al*, 1990), or aggregation of pairs of blastomeres from dissaggregated 4-cell mouse embryos using phytohaemagglutinin (PHA), then treatment with $45\%^{W}/_{V}$ polyethylene glycol (PEG) to induce fusion (Eglitas 1980).

Graham found that the success of the fusion process depended on the time of exposure to the virus and the temperature of the fusion medium, but that the technique yielded

only trophoblastic growth at 8 days of gestation. He fused a diploid blastomere from a dissociated 4-cell embryo to one cell of a 2-cell embryo, which resulted in an embryo with one diploid and one tetraploid blastomere. Liveborn mice were recovered which were found to have no contribution from the tetraploid blastomere. From this he deduced that the inability of the fused embryos to develop was due to their ploidy and not the fusion technique. O' Neill *et al* (1990) were able to recover a small number of early headfold stage tetraploid mouse embryos which were grossly retarded. The remainder were either small embryonic vesicles or merely implantation sites with no embryonic or extraembryonic tissue. These findings seem to agree with those of Tarkowski *et al.* Although blastomere fusion using either inactive Sendai virus or PEG is advantageous over using the highly toxic chemical cytochalasin B, there is still the problem of whether the phenotype of the embryos produced is entirely due to their ploidy or whether the fusigenic agent plays some part in the retardation observed.

More recently, the technique of electrofusion has been developed which causes degradation of the cell membrane following exposure to electric field pulses, allowing adjacent cells to fuse.(Kubiak & Tarkowski, 1985; Kurischko & Berg, 1986; Ozil & Modlinski, 1986; Petzoldt, 1991; Kaufman & Webb, 1990). The advantages of using electrofusion over other methods of producing tetraploids are that, unlike using cytochalasin B, it is not necessary for the cells to be at any particular stage in the cell cycle and by avoiding blocking cell cleavage, the possible problem of generating 2n/4n mosaics is reduced. The embryos are not exposed to toxic chemicals and the time of exposure to the fusigenic stimulus is also very short; in the range of milliseconds. The technique of electrofusion will be discussed more fully in Chapter 3.

1.2 ESTABLISHMENT OF DEVELOPMENTAL LINEAGES IN THE MOUSE CONCEPTUS

The experimental work in this thesis focuses largely upon comparisons between the three primary lineages established early in development. These lineages are (i) the

primitive ectoderm (epiblast), from which is derived the whole of the fetus, the amnion, the yolk sac mesoderm, the allantois and the chorionic mesoderm, (ii) the primitive endoderm, which produces the yolk sac endoderm and parietal endoderm cells of Reichert's membrane and (iii) the trophectoderm which produces the placental trophoblast and the primary and secondary giant cells.

The following description of early mouse development deals with the aspects which are most relevant to the experimental chapters. It is not intended to be a comprehensive review of embryogenesis, which can be found elsewhere (Green, 1966; Rugh, 1968; Hogan *et al*, 1986; Theiler, 1989; Kaufman, 1992)

FERTILIZATION

During fertilization, the female ovum is activated by a male spermatozoon, and the union of male and female pronuclei occurs in the oviduct (Rugh, 1968). The sperm must penetrate the surrounding mass of cumulus cells, to bind to highly species-specific binding sites on the zona pellucida of the ovum. During penetration of the cumulus, the sperm undergoes the acrosome reaction, and releases hydrolytic enzymes. Following the acrosome reaction, the sperm penetrates the zona and the sperm head fuses with the egg membrane. This fusion triggers the series of events known as fertilization, one of the earliest being a change in the surface of the egg preventing the fusion of other sperm. The block to polyspermy in most mammalian species involves modification of both the zona pellucida and the egg plasma membrane (Horvath *et al,* 1993). The zona pellucida block, or 'zona reaction' involves a change in the properties of the zona which prevents further binding or penetration of sperm. These changes are due to biochemical modifications of glycoprotein sperm receptors by enzymes released from egg cortical granules. The second block to polyspermy is the egg plasma membrane block, which is less well characterised and thought to be incomplete in the mouse. This step involves changes in egg membrane components responsible for sperm recognition and changes in membrane fluidity.

Fertilization triggers the second meiotic division and second polar body extrusion (Hogan *et al*, 1986). The male and female pronuclei migrate to the centre of the oocyte and DNA replication takes place. The nuclear membranes break down, the chromosomes assemble on the spindle and first cleavage occurs.

PREIMPLANTATION DEVELOPMENT

After fertilization, the blastomeres of a cleaving mouse embryo are equipotent up until the eight cell stage (Hogan *et al*, 1986). Within a few hours of the formation of eight cells the embryo undergoes compaction to form a morula (Ziomek and Johnson, 1980). The earliest event of compaction is cell polarization, followed by flattening of individual blastomeres, increased contact between blastomeres and the formation of specialized junctions. Polarization results in the development of distinct basal and apical membranes and cytoplasmic domains.

Cell diversification is established by the differential division of polarized 8-cell blastomeres (Pickering *et al*, 1988). Division of 8-cell blastomeres results in either two polar cells or one polar and one apolar cell (the latter is termed differentiative division). There is a higher incidence of differentiative division among the early dividing cells of the morula, which is thought to be a consequence of extensive intracellular contacts. The inside cells of the developing morula which ultimately give rise to the inner cell mass (ICM) of the blastocyst are known to originate usually from one of the first blastomeres to divide, and descendants of this cell tend to divide ahead of the others, which suggests that blastomeres dividing ahead of others may have a selective advantage with respect to becoming inside cells (Spindle, 1982; Graham and Deussen, 1978). Apolar cells resulting from differentiative division are small in size and known to occupy the inside of the morula, while polar cells are larger in size and occupy the outside (Ziomek and Johnson, 1982).

The differentiation process proceeds until a fully expanded blastocyst is formed, which is a hollow ball of trophectoderm cells, arising from outside cells, surrounding a fluid filled blastocoel and small group of ICM (inside) cells. An expanded blastocyst typically has approximately 64 cells, about 20 of which are in the ICM.

During the fifth day of development the blastocyst hatches from the zona and the embryo implants into the wall of the uterus. Trophoblast cells invade the uterus, which reacts by accelerated growth of the decidua (decidual reaction). The decidua capsularis is a thick layer surrounding the embryo and is known to contain many polynucleate cells. As the embryo grows the decidua capsularis becomes thinner. Mesometrial to the embryo is the decidua basalis, which gives rise to much of the placenta. The placenta is formed by fusion of the decidua basalis, the ectoplacental cone, the chorion and parts of the allantois.

ORIGIN OF THE THREE PRIMARY DEVELOPMENTAL LINEAGES

The first diversification of the mouse blastocyst occurs when one group of cells differentiates into the outer trophectoderm, surrounding the fluid filled blastocoel, while the other group of cells differentiates into the inner cell mass (ICM). The inner cell mass differentiates further, into two cell lineages, the primitive ectoderm and the primitive endoderm, so that by the late blastocyst stage, the three primary lineages have been established; the primitive ectoderm, the primitive endoderm and the trophectoderm (Gardner, 1978) (Fig.1.1).

During postimplantation development, the trophectoderm, which differentiates into a typical epithelium, becomes specialised into two subpopulations: the mural trophectoderm and the polar trophectoderm (Hogan *et al*, 1986). The mural trophectoderm is derived from cells surrounding the blastocoel, but not in contact with the ICM. These cells cease to divide, become polyploid and develop into the primary trophoblastic giant cells. Trophectoderm cells in close proximity to the ICM remain

diploid and become the polar trophectoderm, which proliferates after implantation. Some polar trophectoderm cells migrate away from the polar region, around the embryo, become polyploid and join the primary trophoblast giant cells (Copp, 1979). A projection of polar trophectoderm cells penetrates the blastocoel cavity, pushing the ICM ahead of it, forming the extraembryonic ectoderm of the egg cylinder (Fig 1.2). A central cavity develops, fuses with the developing proamniotic cavity of the ICM and after formation of the extraembryonic mesoderm, the extraembryonic ectoderm retreats mesometrially towards the placenta, where it forms the ectoderm of the chorion. Proliferation of the extraembryonic ectoderm mesometrially gives rise to the ectoplacental cone (Rugh, 1968). A proportion of polar trophectoderm cells penetrate the endometrium to form the bulk of the placenta. Some of these cells and cells of the chorion form the secondary giant cells. In the absence of ICM derivatives, trophectoderm cells do not proliferate, but endoreduplicate and become giant (Copp, 1978).

As the primitive ectoderm and extraembryonic ectoderm elongate to form the egg cylinder, the outer primitive endoderm cells differentiate into two cell populations: (i) Visceral endoderm, which is an organised epithelium of cells enveloping both the

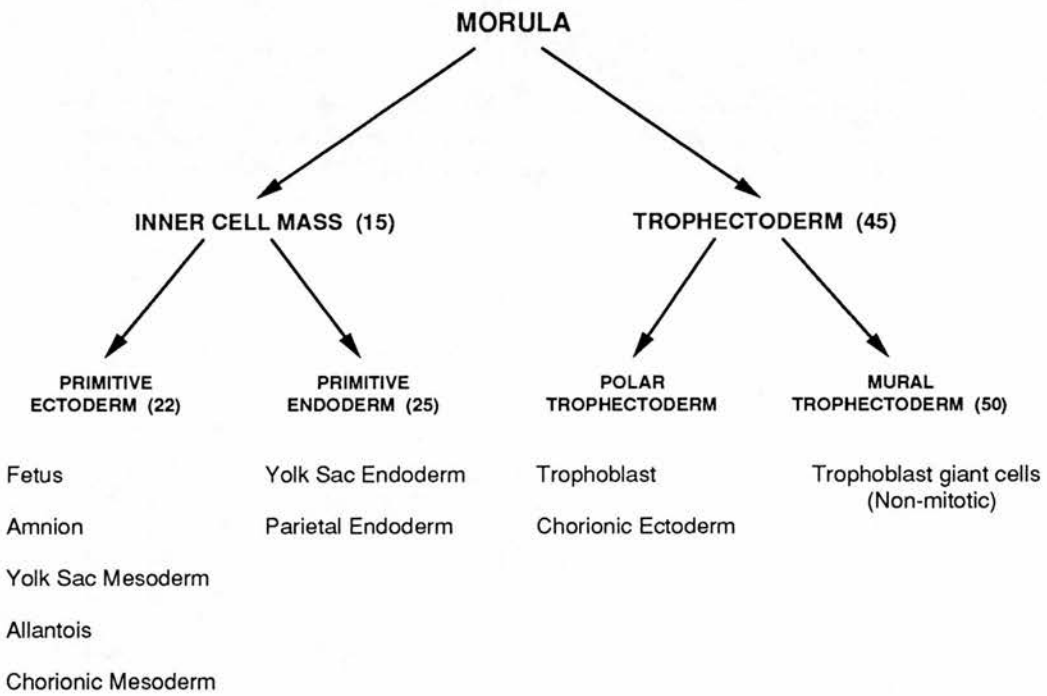


Fig.1.1 Diagram of the lineage relationships (based on Gardner, 1978) showing the developmental fates of the three primary lineages; the primitive ectoderm, the primitive endoderm and the trophectoderm. In this study, the fetus, amnion, yolk sac mesoderm, yolk sac endoderm, parietal endoderm, trophoblast and placenta (largely trophoblast) were analysed. The numbers in brackets indicate the approximate number of cells in tissues at a stage when they appear to be determined, as given by Gardner (1978).

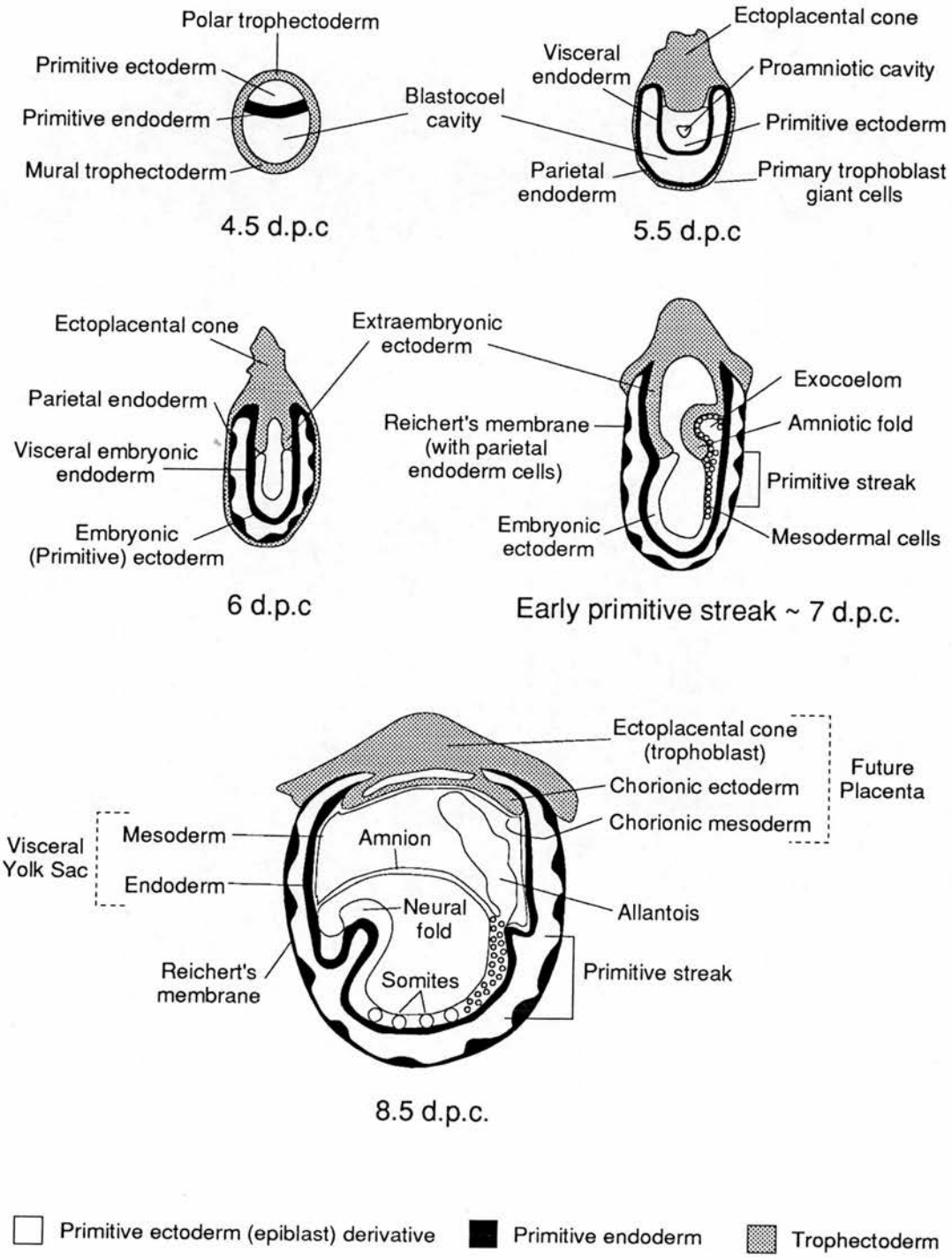
embryonic and extraembryonic ectoderm of the egg cylinder. The extraembryonic visceral endoderm, along with mesoderm derived from the primitive ectoderm (which has emerged from the primitive streak), eventually give rise to the visceral yolk sac. (ii) Parietal endoderm cells, which migrate towards the inner surface of the trophectoderm during implantation, and secrete the components of the thick basement membrane known as Reichert's membrane, which separates the trophectoderm and parietal endoderm. Parietal endoderm cells are migratory and do not form specialized intercellular junctions.

The three definitive germ layers (ectoderm, endoderm and mesoderm; Fig. 1.2) of the fetus are derived by the process of gastrulation at the egg cylinder stage. The core of the inner cell mass (primitive ectoderm) becomes organised into an epithelial layer surrounding the central proamniotic cavity and eventually gives rise to all three germ layers of the fetus, as well as the amnion, yolk sac mesoderm, allantois and chorionic mesoderm (Fig. 1.1). A cavity similar to the proamnion forms in the extraembryonic ectoderm (trophectoderm) and the cavities join to form a narrow lumen. Prior to gastrulation at around 6 days of gestation, a layer of primitive endoderm cells, the visceral embryonic endoderm, underlies the embryonic ectoderm.

Gastrulation

The primitive streak initially forms as a thickening in the embryonic ectoderm (Fig. 1.2), at the junction between the embryonic ectoderm and the extraembryonic ectoderm and the streak subsequently extends anteriorly (Beddington, 1992). The primitive streak establishes the primary axis of the embryo. During gastrulation (around $6^1/_2$days gestation) cells delaminate from the epithelial layer and ingress through the primitive streak to emerge as mesodermal cells. The mesoderm moves proximally into the extraembryonic region of the egg cylinder (between the extraembryonic ectoderm and the primitive endoderm) and laterally in both the

Fig. 1.2 Diagram of early postimplantation development of the mouse embryo (redrawn after Hogan et al, 1986). The different tissues are shaded according to their developmental lineage. The primitive ectoderm derivatives are unshaded, the primitive endoderm shaded black, and the trophectoderm derivatives are grey.


Polar trophectoderm
Primitive ectoderm
Primitive endoderm
Blastocoel cavity
Mural trophectoderm
4.5 d.p.c
Ectoplacental cone
Visceral endoderm
Proamniotic cavity
Primitive ectoderm
Parietal endoderm
Primary trophoblast giant cells
5.5 d.p.c
Ectoplacental cone
Extraembryonic ectoderm
Parietal endoderm
Visceral embryonic endoderm
Embryonic (Primitive) ectoderm
6 d.p.c
Exocoelom
Reichert's membrane (with parietal endoderm cells)
Amniotic fold
Primitive streak
Embryonic ectoderm
Mesodermal cells
Early primitive streak ~ 7 d.p.c.
Ectoplacental cone (trophoblast)
Future Placenta
Chorionic ectoderm
Chorionic mesoderm
Visceral Yolk Sac
Mesoderm
Amnion
Endoderm
Neural fold
Allantois
Reichert's membrane
Somites
Primitive streak
8.5 d.p.c.
Primitive ectoderm (epiblast) derivative
Primitive endoderm
Trophectoderm

embryonic and extraembryonic regions. As the mesoderm forms, the primitive streak elongates distally, finally extending to the tip of the egg cylinder.

Mesoderm emerging from the posterior end of the primitive streak continues to move into the extraembryonic region, where it accumulates and increases in volume, aided by the coalescing of intercellular spaces. Because of this expansion, the proximal rim of the primitive ectoderm and the most distal part of the extraembryonic ectoderm are pushed towards the centre of the amniotic cavity. This bulging at the junction of the embryonic and extraembryonic regions of the egg cylinder, which is more pronounced in the posterior region, results in the formation of the amniotic folds. The amniotic folds eventually fuse to form a continuous layer of extraembryonic mesoderm, with an internal cavity, the exocoelom (Fig. 1.2), which separates the extraembryonic ectoderm from the embryonic ectoderm. Expansion of the exocoelom creates the chorion (extraembryonic ectoderm and extraembryonic mesoderm) and the amnion (embryonic ectoderm and extraembryonic mesoderm).

By the late primitive streak stage (around $7^1/_2$days of gestation), the embryonic part of the egg cylinder surrounds the amniotic cavity. The amnion separates the embryonic region from the exocoelom, the cavity of the future visceral yolk sac. Mesoderm cells which migrate onto the inner surface of the visceral endoderm (of the future yolk sac) differentiate into the first haematopoietic tissue of the fetus. Proximal to the exocoelom is the chorion. The anterior region of the primitive streak, now at the distal tip of the egg cylinder, becomes recognisable as a node. Definitive ectoderm surrounds the node anteriorly and laterally in the dorsal plane of the embryo while the head process and definitive endoderm emerge from it anteriorly, in the ventral layer.

By around $7^1/_2$ days, the mesoderm has formed a complete layer in the embryonic region. Extraembryonic mesoderm continues to be produced from the posterior region of the streak, most of which is incorporated into formation of the allantois. The

allantois starts as projection of mesodermal cells from the posterior margin of the primitive streak. The projection expands proximally, fuses with the chorion at about 8 days gestation and contributes to the placenta, giving rise to the umbilical blood vessels. Mesoderm cells which cover the chorion also contribute to the placenta.

At the headfold stage (around 8 days of gestation), primitive ectoderm continues to invaginate through the streak, to emerge as mesoderm in the posterior region, while organogenesis begins anteriorly. The notochord is derived from the recruitment of cells in the node region, and is continuous with the head processes in the anterior region. As the notochord is differentiating, the embryonic ectoderm anterior to the node and overlying the notochord is induced to thicken to form the neural plate. Elevation of neural folds in the lateral margins of the neural plate lead to the typical 'V' shaped profile of the neurectoderm, with a central neural groove.

There are two well developed sheets of mesoderm on either side of the notochord, which lie between the embryonic ectoderm and endoderm, and are continuous laterally with the extraembryonic mesoderm. Anterior to the node there are two distinct regions of mesoderm: paraxial mesoderm, adjacent to the notochord and lateral mesoderm, adjacent to the exocoelom. The former gives rise to the somites, and the latter to the embryonic coelom. The first somites arise as paired segmental structures anterior to the node. The second pair forms posterior to the first, and so on: eventually about 65 pairs will be formed. As the somites develop from the paraxial mesoderm, the coelom or body cavity develops in the lateral mesoderm. The appearance of the coelom divides the mesoderm into a somatic (dorsal) layer, adjacent to the ectoderm and splanchnic (ventral) layer, adjacent to the endoderm. The coelom extends anteriorly around the foregut, where it develop into the pericardial coelom.

Very soon after the seven-somite stage, the embryo begins the process of turning, which results in the the embryo being curved so that the dorsal surface is on the outer

side of the curve instead of on the inner as it is earlier in development. The turning begins in the head and tail folds, in a clockwise direction along the long axis of the embryo. The midtrunk region begins to turn at around $8^1/_2$ days (11-12 somites). When the embryo begins turning, the yolk sac, after a period of extensive growth, envelops the amnion and greatly enlarged exocoelom. The yolk sac and ectoplacental cone adopt a flattened spherical or disc-like shape, and after growth of the head and tail folds reduces the area of attachment of the trunk region to the yolk sac, the visceral yolk sac and amnion come to envelop the whole embryo.

Placenta

By midgestation the placenta is a complex organ of fetal and maternal tissues. The maternal blood vessels break down, so that the blood cells are in direct contact with the fetal trophoblast. The placenta is a composite of three major tissues types. (i) Trophoblast, which has two distinct layers, the outer spongiotrophoblast closest to the uterine decidual tissue with mainly diploid cells, and the inner labyrinth layer with many polyploid and giant cells. Both layers have maternal blood sinuses and the outer layer is covered with uterine decidual cells. (ii) Extraembryonic mesoderm which gives rise to fetal blood vessels after the allantois fuses with the chorion, and which mingle with the trophoblast cells and maternal blood sinuses in the labyrinth. (iii) Visceral and parietal endoderm cells which are found in the crypts of Duval in the inner placental layer.

1.3 CHIMAERAS AND MOSAICS IN DEVELOPMENT

A chimaera is defined as a composite animal or plant in which different cell populations are derived from more than one fertilized egg, or the union of more than two gametes (McLaren, 1976). A distinction is usually drawn between chimaeras and mosaics, a second class of composite animals. The different cell populations in mosaics arise during development from one egg or a pair of gametes, by way of an

event later on in development. Events which may cause mosaicism, include somatic mutation, somatic recombination and spontaneous or induced non-disjunction. It is often difficult in spontaneous composite animals to determine whether the phenotype is a result of chimaerism or mosaicism. The most studied mosaicism is functional X-inactivation mosaicism, which involves the random inactivation of either the maternal or paternal X chromosome, resulting in a mosaic genotype which may only be recognised in a heterozygous individual.

At the qualitative level there is often little difference between an X-inactivation mosaic and its corresponding experimental chimaera. However, chimaeras are more variable, both between different experimental animals, and between different tissues in a given animal.

Chimaeras can be divided into two categories; primary chimaeras and secondary chimaeras. Primary chimaeras consist of genetically different cell populations which co-exist from a very early embryonic stage, whereas secondary chimaeras can be formed in adults by tissue grafting or transplantation. In the case of the latter only a sub-population of cells or tissues will be chimaeric. This phenomenon is known to occur spontaneously in mammals by transfer of cells between mother and embryo after the onset of embryogenesis. Another class of secondary chimaeras can be produced experimentally to aid the study of transplantation biology. A mouse given a lethal dose of radiation can be rescued by the injection of haematopoietic cells from a genetically different individual.

Although there are many recorded instances of secondary chimaeras produced in invertebrates, attempts to produce primary chimaeras have rarely been successful, because it appears that the determinate development of invertebrates does not allow two embryos to be aggregated and regulated as one individual. In 1961 Tarkowski first reported the successful aggregation of cleaving mouse embryo chimaeras.

Aggregated pairs grew into single large blastocysts, were successfully transferred to foster mothers and developed into morphologically normal animals. The scheme he followed involved removing the zona pellucida mechanically, placing each pair of embryos under oil in drops of culture media to develop to the blastocyst stage and then transferring them to the uterus of a pseudopregnant female. Examination of the newborn retinal pigment confirmed that the offspring were chimaeric. Although aggregation at 8-cell stage is most convenient, embryos have also been aggregated at the two cell, four cell and morula stage. After blastocyst formation embryos will no longer aggregate. A few years later Mintz (1962, 1967) independently described a more manageable method of producing aggregation chimaeras.

Primary chimaeras can also be produced by injecting individual or groups of cells into the blastocoel of a genetically different blastocyst. The injected cells may either be from a dissociated blastocyst or embryonic stem cells. Injected cells become incorporated in the inner cell mass. Gardner first described a method in 1968 in which he introduced blastocyst cells dissaggregated in calcium and magnesium free saline into a blastocyst then transferred them to the uterus of a psuedopregnant female. Gardner also reported in 1971 that the whole ICM can be successfully transferred surgically to the blastocyst. Aggregation with the host ICM gave rise to a chimaeric embryo.

Chimaeric embryos have two important uses in developmental biology. Firstly, by studying the fate of two marked cell populations in a chimaera, it is possible that the origin of tissues and cell lineages during development may be traced. A few genetically marked cells injected into a blastocyst (Gardner and Papaioannou, 1975) can give insight into the fate of the descendants of those cells later in development. Secondly, by creating a chimaera which has one population of cells with a mutant allele and one which is wild type at that locus, studies of recessive lethal mutations can be carried out and new mutation created. Mutant cells are rescued and maintained by

the normal population, allowing both the developmental potential of the abnormal cells and the phenotypic effects of the mutation to be studied. In the field of developmental genetics chimaeras can be used to analyse how genetically different cells interact to form an adult animal, and whether the phenotype of each individual cell is determined solely by its own genotype, or whether it may be influenced by its cellular environment.

Aggregation chimaeras have provided many insights into the developmental potential of embryos with various types of developmental defects. Experimental embryos which have been studied include androgenetic, parthenogenetic and gynogenetic embryos (Nagy *et al.*, 1987, 1989; Surani *et al.*, 1987, 1988; Thomson and Solter, 1988, 1989; Fundele *et al.*, 1989, 1990, 1991), embryos with various chromosome anomalies (Epstein *et al.*, 1982, 1984; Cox *et al.*, 1984; Fundele 1985) as well as embryos with lethal single gene defects, such as various t-haplotypes (Mintz, 1964; Bennett, 1978) and lethal yellow (Barsh *et al.*, 1990). One advantage of using aggregation chimaeras is that both cell populations may contribute to the fetus and all of the extraembryonic tissues. Although, in many cases investigations have been limited to fetal or adult tissues, in some cases the placenta (Cox *et al.*, 1984; Epstein *et al.*, 1984) and other extraembryonic tissues (Nagy *et al.*, 1987; Surani *et al.*, 1987, 1988; Thomson and Solter, 1988, 1989) have also been considered. This has provided additional valuable information about the developmental potential of the abnormal cell type, particularly in the case of cells from androgenetic, parthenogenetic and gynogenetic embryos.

In this thesis mouse aggregation chimaeras were used to study the developmental potential and fate of tetraploid cells in a tetraploid↔diploid chimaeric environment. The ultimate aim of the study was to determine whether tetraploid cells were able to contribute equally to all tissues, embryonic and extraembryonic, of chimaeric mouse embryos studied at $12^1/_2$ days of gestation, and if not, which tissues they persisted in.

The details of this aspect of the thesis are discussed in Chapter 5. Another major part of this thesis involved the analysis of normal diploid↔diploid chimaeric conceptuses to set a base line for the interpretation of experimental chimaeras, which have one normal and one abnormal (*e.g.* tetraploid) cell population. The data from these experiments are discussed in Chapters 4 and 5.

In *Drosophila* mosaics, the distribution of cell populations is easy to interpret. If two non-identical cells arise at the first cleavage, the resulting fly is half one cell type and half the other. Likewise if the distinct cell arises later in development, its progeny forms a cohesive spatially contiguous patch of cells in the adult fly. The distribution of cell populations in mammalian chimaeras is much more difficult to interpret, since the cells mingle and cell movement occurs, as does cell selection and cell death. Analysis of visible mosaic patterns in chimaeric tissue relies heavily on the selection of a cell marker suitable to the type of analysis to be performed, no single marker is suitable for both qualitative and quantitative analysis. The cell markers used in this study are discussed in Chapter 2.

1.4 AIMS OF THE EXPERIMENTAL WORK

The overall aim of this thesis was to create aggregation chimaeras between diploid embryos and tetraploid embryos, and to determine whether tetraploid cells were capable of colonising all developmental lineages of mouse conceptuses analysed at $12^1/_2$ days of gestation.

Chapter 2 outlines the genetic cell markers which were considered to allow analysis of chimaeric conceptuses, along with a description of the advantages and shortcomings of those markers eventually used in the study. The method of producing tetraploid embryos by electrofusion is detailed in Chapter 3. The efficiency of the technique as well as the efficiency of detecting tetraploid cells in histological sections is discussed. Chapter 4 describes the production of a series of diploid↔diploid control chimaeras to

form a baseline for the analysis of the tetraploid↔diploid chimaeras. The production of the tetraploid↔diploid chimaeras, and the fate of tetraploid cells in the chimaeric conceptuses is discussed in Chapter 5. The physical dimensions and the morphology of the tetraploid↔diploid conceptuses is compared to diploid↔diploid conceptuses in Chapter 6.

The findings from Chapter 3 were published, in collaboration with Prof. Matthew Kaufman, Dr. Sheila Webb and Dr. John West; Electrofusion of mouse embryos results in uniform tetraploidy and not tetraploid/diploid mosaicism. *Genetical Research* (1992), **60**, pp185-194.

Data from Chapter 4 is included in; Quantitative analysis of mid-gestation mouse chimaeras: non-random composition of the placenta, co-authors; Jean Flockhart, Margaret Keighren and Dr. John West. Roux's Archives of Developmental Biology, in press.

CHAPTER 2
GENETIC CELL MARKERS FOR MID-GESTATION CHIMAERIC CONCEPTUSES

2.1 INTRODUCTION

This chapter is intended to provide an overview of the types of cell markers suitable for the analysis of mid-gestation chimaeric conceptuses.

The criteria for the ideal marker have been described by others (McLaren, 1976; West, 1984). It is important that the cell marker should be stable, cell localised and autonomous and that there should be no extracellular excretion or transfer of the marker between cells. An ideal cell marker should also be heritable and ubiquitous both throughout the embryo and throughout development. It is also important that the marker is developmentally neutral, so that selection or non-random association does not occur. Several genetic variants of a given cell marker, characterising different strains is also useful. Detection of the cell marker should be easy.

Not all of the points outlined are relevant to the study of the mid-gestation fetus and membranes, and cell markers must be chosen to suit the tissue to be analysed, as well as yield the information required.

Several markers with different strengths and weaknesses were chosen to be incorporated into chimaeric embryos. Since the vast majority of the analysis took place at $12^1/_2$ days of gestation, it was not possible to use exogenous labels such as tritiated thymidine, which would have been diluted out during cell division, and so are better suited to studies earlier in development. To overcome this problem, the markers chosen were all genetic cell markers.

Eye pigment was the simplest genetic cell marker to use, as it requires no reagents for detection and can be viewed in wholemounts or sections. Although potentially both quantitative and spatial, pigment is only useful for studying the eye in mid-gestation chimaeras. Because of this, pigment cannot be used as a comparison between tissues. However it may be used to compare the spatial distribution between groups of chimaeras.

The bulk of the study focuses on the quantification of the proportions of two electrophoretic variants of the enzyme glucose phosphate isomerase (GPI-1) in homogenated tissues. GPI-1 was chosen because the enzyme is relatively stable and expressed in all tissues of interest in the $12^1/_2$ day mouse conceptus. The advantage of using GPI-1 is that the detection technique is fairly simple, accurate and reproducible. The assay requires only small samples. Also because there are several electrophoretic variants of the enzyme, both cell populations are assayed at the same time. The main drawback with using a biochemical marker is that in order to assay for enzyme activity, the tissue being studied must be homogenized. Because of this the information gained from GPI-1 electrophoresis is quantitative and it is difficult to obtain detailed spatial analysis *in situ.*

To enable qualitative analysis, the ß-globin transgenic marker was chosen. The presence of the transgene in cells is detected by *in situ* hybridization, and a great deal of information can be gained regarding the spatial arrangement of marked cells in histological sections. *In situ* hybridization can also be used for quantitative analysis. There is, however, the problem of overlapping and unlabelled cells in sections. Overlapping nuclei make the identification and scoring of individual cells difficult. Unlabelled cells may be the consequence of a poor identification technique or may be due to the absence of marker in that particular section. With thin sections, it is possible for the *in situ* target sequence to be excluded from the section and the section to be scored as a false negative. This marker was used for the evaluation of tetraploid

production by electrofusion (see Chapter 3), and for the analysis of some tetraploid↔diploid yolk sacs (see Chapter 5).

The *lac-Z* transgenic marker was a relatively newly established cell marker when this thesis was planned. It is a cytoplasmic marker and it was hoped that it would be possible to use this cytoplasmic marker either alone, or in conjunction with the ß-globin nuclear marker. Cytoplasmic markers are not affected by section thickness. By labelling one component with the ß-globin transgene and the other with the *lac-Z* transgene it should have been possible to overcome the problem of false negative cells in histological sections. Unfortunately the *lac-Z* transgene could not be maintained in the required mouse strain and the marker had to be abandoned

A variety of markers were employed during this study, as detailed below, although the majority of the data analysed was from the analysis of GPI-1 in tissues of $12^1/_2$ day conceptuses. Each method yielded a different set of data which were merged together to provide a good idea of how the two cell populations were behaving in aggregation chimaeras.

2.2 PIGMENT

Pigment was one of the first markers used to analyse chimaeras. Tarkowski studied the pigmented retinal epithelium of chimaeras in 1964. Although restricted to a small group of pigmented tissues, pigment fulfils many of the other criteria for an ideal cell marker (West, 1984). Genetic variation in pigmentation is easy to detect as the presence or absence of melanin granules. The most commonly used variant of pigmentation is the albino mutation (c) which caused failure of synthesis of melanin due to a deficiency or alteration in the structure of tyrosinase. Pigmented cells are localised in the pigmented epithelium of the retina and are easily seen in embryos at $12^1/_2$ days of gestation in both wholemounts (Fig. 2.1*a* & *b*) and sections. The pigmented epithelium is a monolayer of cells which appears as a 1-dimensional string

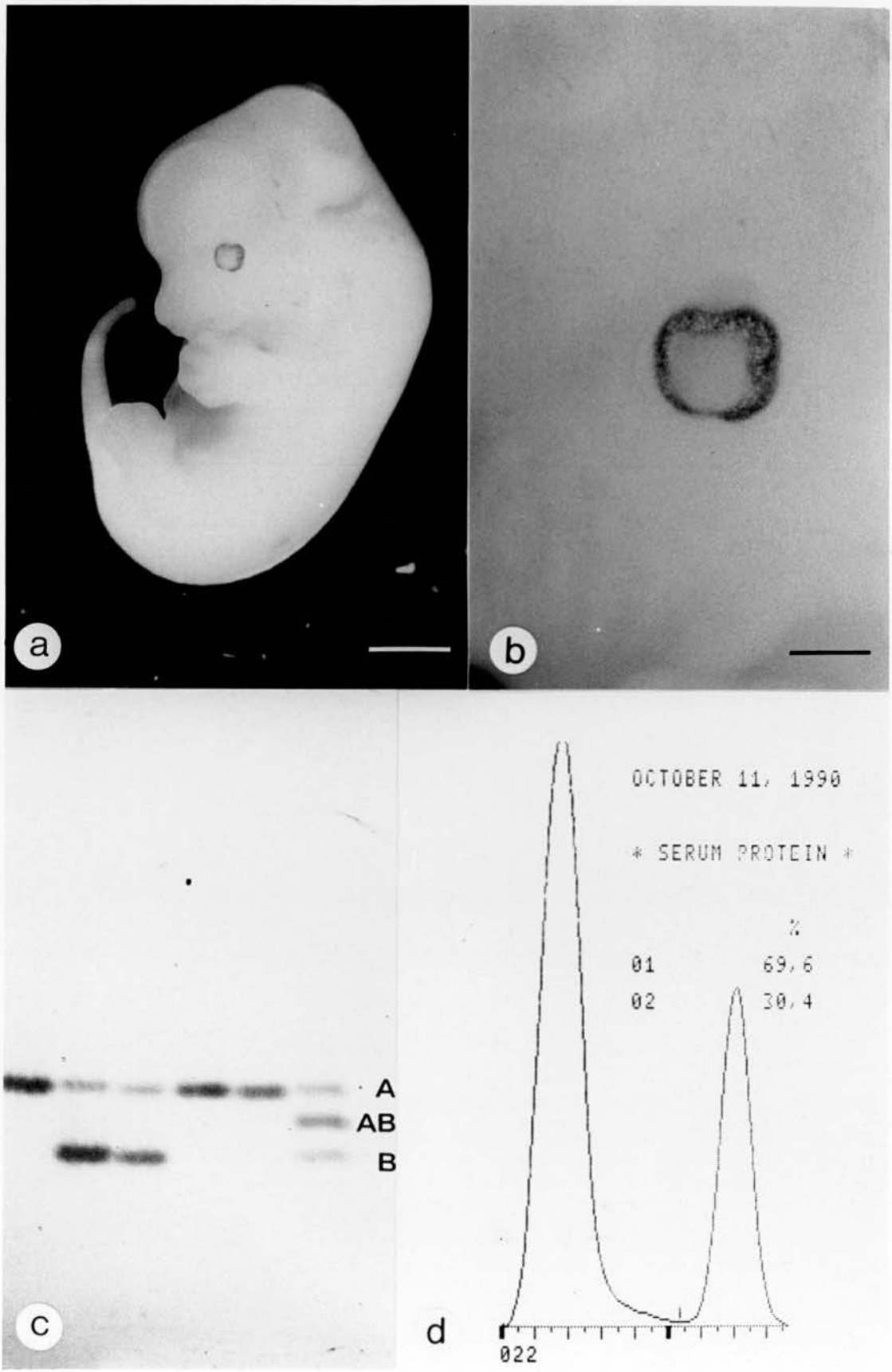
in histological sections (McLaren, 1976). Because of this, analysis of the pigmented epithelium yields easily detectable spatial information and data on clone size (West, 1976). However pigment as a marker is limited to the retinal epithelium, the membranes of the inner ear and other structures in the eye. Pigment in the coat is not such a precise marker since each hair follicle may have two independently derived populations of melanocytes. Pigment is secreted into the hair by melanocytes, which are derived from the neural crest (McLaren, 1976).

2.3 GLUCOSE PHOSPHATE ISOMERASE

It is possible to use genetic biochemical variants as an indirect assay of the proportions of contributing cell types in aggregation chimaeras (West, 1984). Many biochemical markers exist, such as haemoglobin and ß-glucuronidase. One advantage of using biochemical variants is that many can be separated electrophoretically (glucose phosphate isomerase, isocitrate dehydrogenase) and the marker from both cell populations can be assayed in the same sample. After electrophoresis the proportions of the cell populations can be estimated using scanning densitometry.

The enzyme glucose phosphate isomerase (GPI-1) (E.C. No. 5.3.1.9., phosophoglucose isomerase) is the most commonly used biochemical marker for studies with chimaeric mice. It is a dimeric enzyme which catalyses the interconversion of glucose-6-phosphate and fructose-6-phosphate. Three monomer types have been identified; GPI-1A, GPI-1B and GPI-1C. Heterodimers are possible which can be resolved by gel electrophoresis (DeLorenzo and Ruddle, 1969). GPI-1 is a useful marker since it is expressed in all body tissues. The assay system is sensitive enough to detect a minor component of around 1-2% of the enzyme. There are also disadvantages to using a biochemical marker, not least that the assay is indirect and

Fig.2.1 *(a)* Mouse fetus at $12^1/_2$ days of gestation (bar is 2000μm). *(b)* Eye of the $12^1/_2$ day fetus at higher power showing the presence of pigmented cells in the retinal epithelium of the eye (bar is 500μm). *(c)* Photograph of acetate gel plate after running homogenized tissue samples and staining for GPI activity. A control AB blood sample run along side the samples shows A, AB and B bands in a 1:2:1 ratio. The bands migrate from the cathode, the B band migrating faster. *(d)* Printout from the Helena Process-24 gel scanner, after scanning through the B, then A band of a tissue sample on a acetate gel. The proportion of GPI-1B in the sample is estimated at 69.6% (the higher peak) and GPI-1A at 30.4% (lower peak).


a
b
A
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01 69,6
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allows no indication of the spatial arrangement of the cell populations. Also the proportions are the average value of the whole sample, and may include contamination of blood, or different cell types within the one tissue.

GEL ELECTROPHORESIS

Method

The method of enzyme electrophoresis used in this study was described previously by (West and Green, 1983; West *et al*, 1986). Electrophoresis was carried out using electrophoresis tanks and Super Z-12 applicators obtained from Helena Laboratories and a Consort Bioblock Scientific power pack. Titan III cellulose acetate plates (Helena Laboratories) were soaked in electrophoresis buffer (see appendix IV) for 30min The tank reservoirs were filled with electrophoresis buffer and Whatman filter paper was used to form wicks (Hebert, 1989). Samples were applied to blotted plates and run from anode to cathode at 200v for 60min, then stained (see appendix IV) for up to 20min on a 37°C hotplate. After rinsing in water the plates were fixed in 5% acetic acid for 5min then washed in distilled water for 15min.

The resulting bands on the gel plate (Fig.2.1*c*) were scanned dry, without clearing in oil, using a Helena Process-24 gel scanner to determine the relative proportions of GPI-1A and GPI-1B in the samples, which gives an estimate of the relative proportions of the two cell types in the sample (Fig.2.1*d*).

Assessment of Staining Linearity

The accuracy of the staining technique was tested using samples of known proportions of GPI-1A and GPI-1B cell types. This was achieved by mixing mouse kidney homogenate from a mouse strain homozygous for GPI-1A with that of a strain homozygous for GPI-1B. Proportions ranging from 100% A tissue:0% B tissue, through 50%:50% to 0% A:100% B were used. The bands were separated by gel

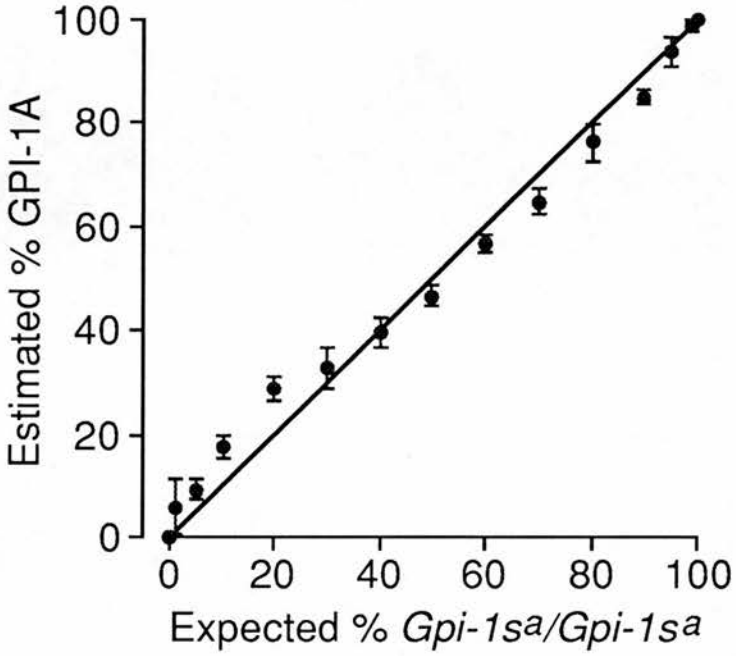


Fig.2.2 Relationship between the percentage of *Gpi-1s*^{a}/*Gpi-1s*^{a} tissue (by weight) in a series of kidney homogenates and the %GPI-1A (mean ± S.E.M. of three determinations) estimated by electrophoresis and scanning densitometry. The line shows the expected linear relationship if GPI-1A and GPI-1B have equal activities.

electrophoresis and scanned to compare the known percentage of tissue in the sample mixture with the percentage of each GPI type detected on the gel. A graph was plotted of the expected percentage of GPI-1A in the kidney homogenate sample against the mean percentage of GPI-1A detected in the samples by the scanner (Fig. 2.2). The plot tends towards a straight line, which indicates that the the relationship between the expected percentage and the mean detected percentage of GPI-1A is directly proportional. There was a small deviation from the expected value when there was a large difference between the percentages of each tissue type, the minor band being slightly overestimated. The reason for this is that when the minor band is a small proportion compared to the major band there is the possibility that the reaction occurring with the enzyme GPI in the major band will have reached saturation point, while the minor band continues to intensify. Therefore, when scanned, the minor band is overestimated. The conclusion can be drawn that the staining is slightly more accurate when the proportions of sample tissue are closer in value (Markert and Masui, 1969).

2.4 ß-GLOBIN TRANSGENE

The use of chimaeras for the study of cell lineage analysis has been limited by the lack of a suitable genetic cell marker which could be visualised in histological sections. The first use of a ubiquitous *in situ* cell marker was reported by Rossant *et al* (1983), where species specific satellite DNA probes were used *in situ* to distinguish the cell populations in *Mus musculus* ↔*Mus caroli* chimaeras. This system may not be ideal, since there is the concern that interspecific chimaeras do not undergo cell mixing to the same extent as intraspecific chimaeras. If this were a problem then the data could not be extrapolated to explain events during normal development.

More recently, the advent of transgenic mice has seen the insertion of an exogenous gene into a live animal. One transgenic mouse strain which has become widely used

was produced by Lo (1983) The strain, 83, has approximately one thousand copies of the mouse ß-major globin gene tandemly integrated into one site on chromosome 3. Most of the criteria laid down for the ideal cell marker are fulfilled by the ß-globin transgenic marker. The marker is ubiquitous in nucleated cells, is stably inherited, not secreted and is not influenced by the environment. (Thomson and Solter, 1988). However, there is the potential problem that a large, exogenous, though unexpressed region of DNA inserted into the genome may not be developmentally neutral.

Another important factor is the ease of detection of the transgenic marker. The methods described for detection of the transgene by *in situ* hybridization (Thomson and Solter; 1988 and below) are labour intensive procedures, but do produce reliable results. One aspect of the project attempts to assess how efficient the technique of *in situ* hybridization is, using the methods and conditions described in this study and to estimate the percentage of unlabelled cells found in histological sections where every cell is expected to be labelled (see Chapter 3).

Section thickness has a crucial bearing on the percentage of nuclei scored as positive after *in situ* hybridization. In sections which are thin compared to the nuclear diameter, some cells will lack a section of nucleus, creating a higher incidence of false negative results. In sections that are too thick, nuclei overlap and scoring becomes difficult (Thomson and Saltor, 1988). Section thickness is therefore a compromise between achieving the greatest number of positive nuclei per slide and having the least number of overlapping cells. Using mouse strains which are homozygous for the transgenic marker is advantageous in that homozygous strains will have two hybridization signals per nucleus compared to one spot per nuclei in hemizygous strains. When there are two hybridization sites per nucleus, there is less chance that both target sequences will be excluded from the section than one sequence being excluded from a hemizygote.

DNA Probe

The DNA probe, pMßð2, used to detect the ß-globin sequences in the strain 83 derived cells of the samples was provided by Dr. John Ansell. Institute of Cell, Animal and Population Biology, University of Edinburgh. The probe plasmid pMßð2 was derived from the plasmid pMBJ, which was inserted into the transgenic strain 83 mice (Lo, 1983). The plasmid was linearised using the restriction enzyme *Eco* RI and labelled by random primed incorporation of digoxigenin-labelled deoxyuridine-triphosphate, using a Nonradioactive DNA Labelling and Detection Kit (Boehringer Mannheim). Routinely 1µg was labelled using the kit reagents to produce a stock concentration of 20ng/µl (see appendix V).

Hybridization

Hybridization procedures were modified (Keighren and West, 1993) from those of Morris *et al*, (1990) and Dr. Reinhald Fundele (personal communication). Before performing *in situ* hybridization, the slides were dewaxed by immersion in Histo-Clear, rehydrated through a series of graded alcohols, then washed in PBS. Endogenous peroxidase activity was inhibited by immersion for 30min in 3% hydrogen peroxide in methanol. The DNA present in the sections was denatured using 1mM NaOH at 70°C, followed by PBS at 4°C (see appendix V). The probe was denatured by boiling for 10min, then removing to ice for a further 5min. After prehybridization for 15 min at 60°C in a humid box, hybridization took place under sealed hydrophobic coverslips (Gel Bond), overnight at 60°C, using the ß-globin probe at a concentration of 20ng/slide (see appendix V).

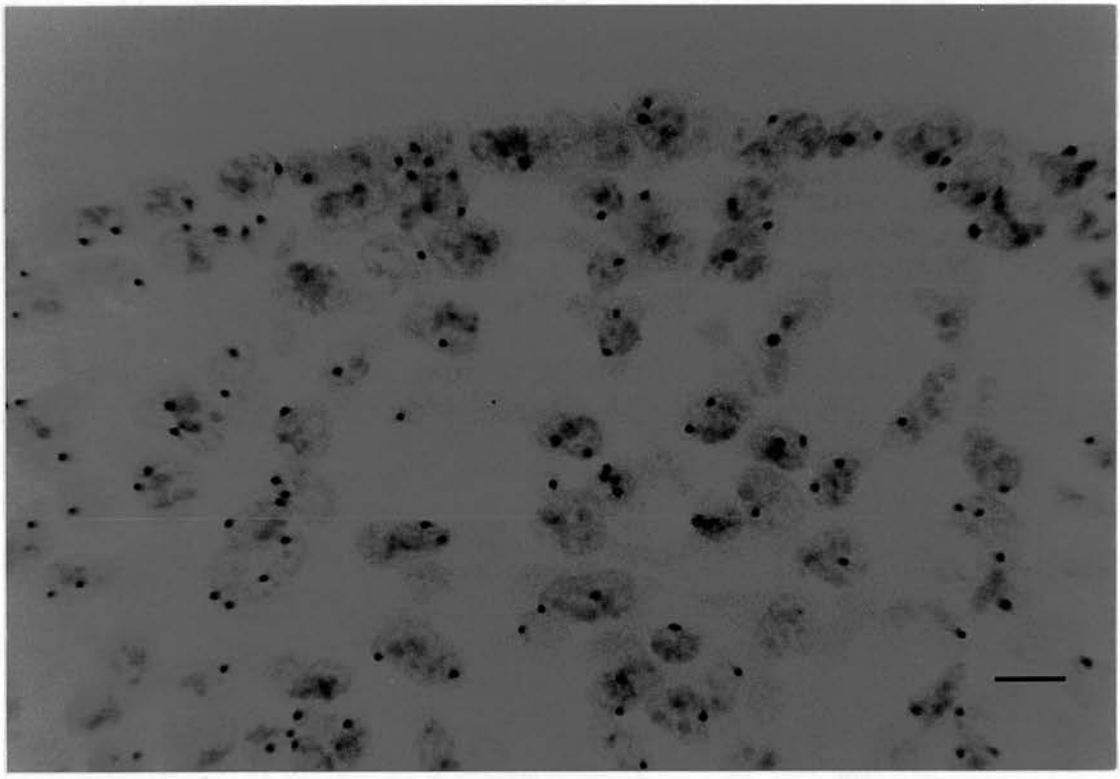


Fig 2.3 Embryonic tissue after *in situ* hybridization to the ß-globin transgene. Hybridization is identified by the presence of dark spots in the nuclei. In samples of tissue from diploid individuals homozygous for the ß-globin transgene there are two spots per nuclei (as in this case), one spot per nuclei in hemizygous tissues (bar is 10μm).

Detection of Hybridization Signal

After hybridization the slides were washed in a series of solutions of SSC with 0.1% Triton-X detergent of increasing stringency (see appendix V), and then with 5% BSA added to block non-specific conjugation of antibody used to detect the digoxygenin labelled probe. The antibody used (Boehringer Mannheim) was an anti-digoxygenin antibody from sheep, Fab fragments, conjugated with horse-radish peroxidase (HRP). After conjugation of the anti-digoxygenin antibody (see appendix V) the slides were washed and flooded with the development reagent which contained the peroxidase substrate diaminobenzidine. The product of the reaction is a brown, water and ethanol insoluble precipitate, which can be viewed under either transmitted or phase contrast light microscopy. The sections were counterstained with haematoxylin and eosin (see appendix), dehydrated through graded alcohols, followed by immersion in Histo-Clear, before being mounted with Histo-Mount (National Diagnostic) (Fig. 2.3).

Quantification of Hybridization Signal

It was possible to quantify the ß-globin marker in histological sections by microscopy. Areas of tissue to be scored were delineated using an eye-piece with a 10x10 grid. Using a x25 objective and x12.5 eye-piece, the cells within a single square, corresponding to 50µm x 50µm on the slide, were scored as either unscoreable, no signal or 1 or 2 signals (depending on the strains used). The method of quantification of the hybridization signal and analysis of the resulting data is discussed in Chapter 3.

2.5 *lac Z* REPORTER GENE

No cell marker is ideal in every respect to follow the fate of one cell component in a chimaeric embryo. An alternative marker was sought to use in conjunction with the ß-globin transgenic marker, to facilitate identification of both cell types in a chimaera. The marker which was considered was a transgenic strain carrying the *E.coli lac Z* gene, under the control of a rat ß-actin promoter (Beddington *et al*, 1989), which is a

cytoplasmic marker. Cytoplasmic markers have an advantage over nuclear markers in that they are not compromised by section thickness and may be used on whole mount preparations, facilitating greatly the analysis of clones in the intact embryo. Expression of the the *lac Z* transgene is apparently ubiquitous and cell autonomous in the inner cell mass derivatives, although activity has not been seen in trophectoderm or primitive endoderm derivatives. Constitutive expression is confined to embryos of 10 days of gestation (Fig 2.4*a*; photograph by kind permission of Dr. R. Beddington), staining before 10 days being punctate, and downregulation of the transgene occurring from day 11 or 12.

TRANSGENIC STOCK

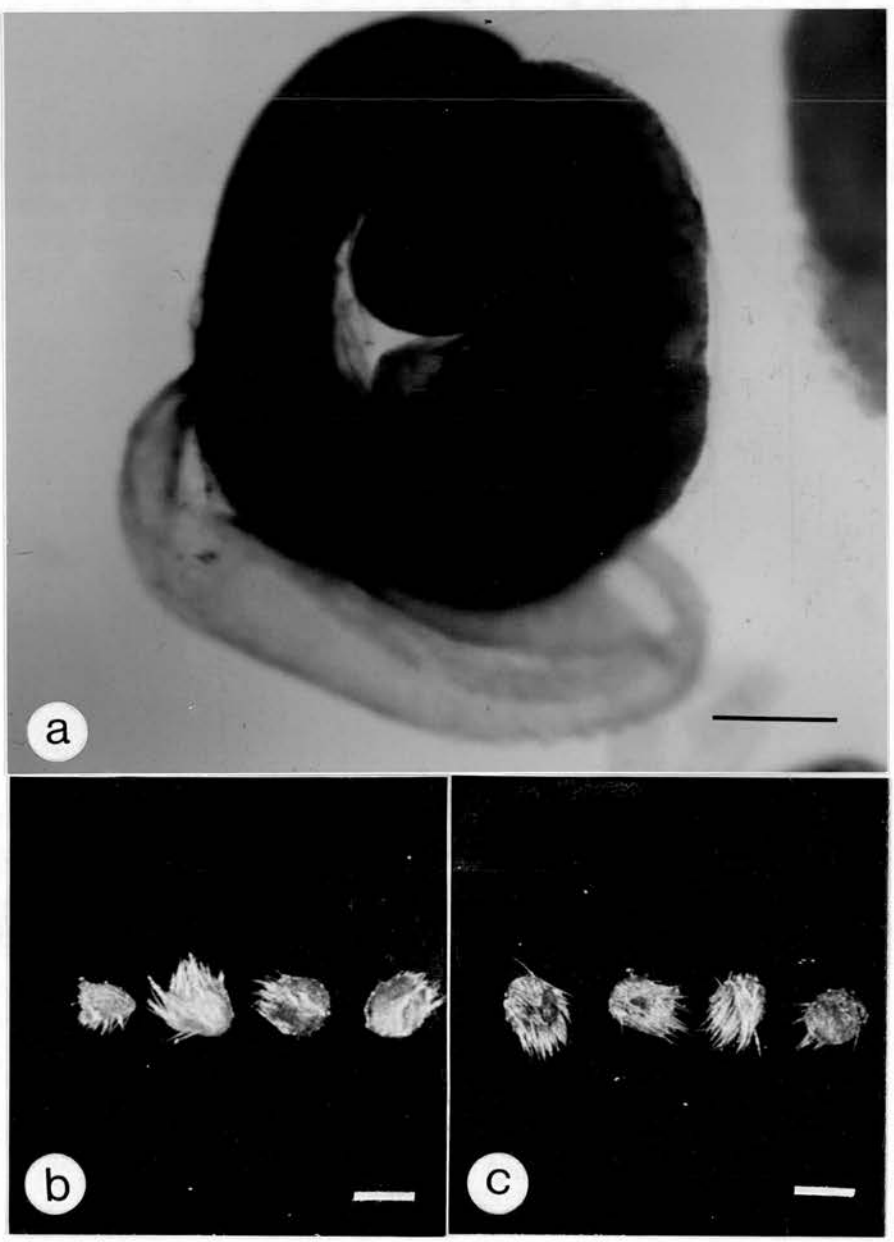
The transgenic mice used, *Tg(Act-lac Z)-1*, were kindly supplied by Dr. Rosa Beddington (Sir William Dunn School of Pathology, University of Oxford), and were an albino outbred strain (PO), carrying the *E. coli lac Z* gene under the control of the rat ß-actin promoter. The transgenics had been produced by injecting the 4.3kb *Bgl* II/*Sca* I fragment of the plasmid *pIRV-Neo-Act-lac-Z*, which contains the *E. coli lac Z* gene fused to the ß-actin promoter (Beddington *et al*, 1989).

The transgenic stock was heterozygous for the transgene, and was crossed on to the congenic albino strain BC (see appendix XI) in Edinburgh to produce the stock termed ACTA. The offspring of the crosses were screened for expression of the transgene.

Identification of Transgenics

Putative transgenic progeny were screened for *E.coli ß*-galactosidase activity by means of an X-Gal staining procedure (see appendix X). Ear punches were taken around weaning age, 21 days and fixed in gluteraldehyde fixative. The samples were then washed and stained with X-Gal, in the dark. The punches were scored and individuals

Fig 2.4 *(a)* 10 day conceptus after staining for ß-galactoside activity (by kind permission of Dr. Rosa Beddington). The enzyme activity is identified in the fetus, but not the yolk sac, by blue staining in the presence of X-Gal. *(b)* and *(c)* Ear punches from weaning age mice, stained with X-Gal to indicate the presence of ß-galactoside activity. *(b)* shows no blue staining in the ear punches, which were scored as negatives. *(c)* blue staining ear punches scored as positives (bar is 100μm in *(a)* and 2000μm in *(b)* and *(c)*).


a
b
c

with the most darkly staining blue punches were used in further crosses with BC stain mice.

Transgenic Embryos

Control embryos were generated by crossing BC females and ACTA males along with the reciprocal cross, and were collected at $9^1/_2$ days of gestation. The embryos were treated as for the ear punches (see appendix X). After recording the staining the embryos were refixed in gluteraldehyde, dehydrated, and embedded in paraffin wax (see appendices X and VII).

RESULTS

Typing the ACTA Strain

After staining, the ACTA progeny ear punches were scored according to the strength of the blue colour produced by the X-Gal stain. The categories were: - (no evidence of blue staining; Fig 2.4*b*) and +, ++, +++ (with increasing intensity of blue staining; Fig. 2.4*c*). Individuals which scored highly positive were retained for further breeding whilst the negatives were discarded. Breeding pairs were set up with BC mice using either ACTA males or females. From the original four transgenic males received from Pathology, Oxford, a total of 18 pairs were established over a period of approximately six months. After three generations, the extent of staining in the ear punches was greatly reduced, even when left to stain in excess of 48hr and the incidence of negative punches increased.

ACTA Control Embryos

Initially, to test the embryo staining method, a cross was set up between a ACTA male and a wild type female, to generate $9^1/_2$ and $10^1/_2$ day embryos. After staining the embryos were sectioned at 4μm (see appendix VII) to determine the spatial distribution of the X-Gal staining. The pattern of staining was in accordance with that reported by Beddington *et al* (1989), confined to the inner cell mass derivatives. However, when

second generation ACTA mice were crossed with albino wild type, to produce embryos at $9^1/_2$days of gestation, the reciprocal to the expected staining pattern was observed, ie. no staining in the embryo but blue staining in the yolk sac endoderm. Initially the unexpected staining pattern was thought to be maternal contamination in the yolk sac, but the pattern was also seen when the transgenic component was paternally inherited. Since at this time in the experiment, the extent of X-Gal staining was reduced considerably, the embryos were left in stain for up to four days, so that the yolk sac staining may have been an artefact of overstaining. To verify this fact a series of $9^1/_2$ day albino embryos which did not carry the transgene were subjected to the X-Gal staining procedure. After a comparable time in the X-Gal stain, a similar pattern of staining was seen in the yolk sacs of the albino embryos, confirming that the unusual staining was non-specific and due to excessive exposure to the stain.

DISCUSSION

After considering the results obtained from the routine ear punches it was decided that the ACTA stain was too inconsistent to be used as a chimaeric marker. There are several possible explanations for the failure of X-Gal staining with subsequent generations, the most obvious being the failure of reagents. All of the reagents were renewed several times during the course of the experiments. Alternatively the *E.coli lac Z* transgene was being switched off or lost completely due to some interaction with the genetic background it was being bred on to, by virtue of methylation of the promoter, for example. In fact, previous attempts by Beddington (personal communication) to breed the transgene onto a C57BL genetic background produced poor results and loss of *ß-galactosidase* function. It was also confirmed that spurious staining of the yolk sac may occur with prolonged exposure to the X-Gal stain. Since the transgene was reported to be expressed constitutively in the primitive ectoderm derivatives only at 10days it was not considered to be ideal for the study of chimaeras, since it would not have been possible to compare the primitive ectoderm derivatives

with the primitive endoderm and trophectoderm derivatives. Further attempts to develop a Lac-Z marker stock were abandoned in favour of continuing with other markers. It remains the case that no constitutive ubiquitous promoter has yet been found to work out of context in the genome.

2.6 CONCLUSION

In this study several different genetic cell markers were used to identify the cell populations contributing to the aggregation chimaeras which were generated. The presence of melanin granules was used in the pigmented retinal epithelium of $12^1/_2$ day conceptuses to confirm whether the embryos were chimaeric and to give a rough indication of the proportion of each cell type present.

GPI gel electrophoresis was used to more accurately determine the contribution of each cell component to the chimaeras at $12^1/_2$days. This method was very useful in detecting small contributions of one cell type, and was a reliable and reproducible assay to perform. A major advantage of this technique was that it was possible to assay small tissue samples accurately.

In situ hybridization to the ß-globin transgene in histological sections was employed to analyse spatially the arrangement of tetraploid cells in certain tissues of the $12^1/_2$day tetraploid↔diploid chimaeras, as well as to test the ability of the electrofuion technique to produce non-mosaic tetraploid embryos. The efficiency of *in situ* hybridization is governed largely by the section thickness which is used. Because not every nucleus in thin sections will have a target sequence, a percentage of cells will always be scored falsely negative. For this reason *in situ* hybridization is not ideal in cases where the smaller population in chimaeras is the unlabelled one. The section thickness chosen, therefore is a compromise between achieving maximum signals per section and the ability to distinguish between overlapping nuclei.

The *lac-Z* transgenic marker was to be used in conjunction with the ß-globin *in situ* marker, so that one cell population would be marked cytoplasmically, and the other would show hybridization signal in the nucleus after both techniques had been performed. Marking each cell population would have eliminated any confusion over genuine and falsely labelled cells. However the *Lac Z* strain was not well suited to the system and was abandoned before any experimental data was collected.

In conclusion three separate genetic markers were employed in this study; the distribution of pigment in the retinal epithelium, the proportion of GPI-1 in the tissues and the visualisation of the ß-globin transgene in histological sections. Each method has allowed assessment of the experimental conceptuses in a different way. Although, in reality, a limited amount of *in situ* data was gathered and the bulk of the data was the result of electrophoretic analysis, the sum of the three systems allowed a detailed quantitative picture of how the two cell populations behave in both diploid↔diploid and tetraploid↔diploid chimaeras, and provided insights into the spatial distribution in certain tissues, such as the eye and the yolk sac (see Chapters 4 and 5 respectively for details).

CHAPTER 3.
TETRAPLOID EMBRYOS GENERATED BY ELECTROFUSION

3.1 INTRODUCTION

Previously cell fusion was achieved *in vitro* by using chemicals or inactivated viruses (See Chapter 1.1). Methods often employed membrane disrupting agents under non-physiological conditions. Zimmermann *et al* reported a new fusion technique in 1980 (for review see: Zimmermann and Vienken, 1982), based on the exposure of cells to an alternating, non-uniform electric field, followed by the application of a short electric field pulse of high intensity. This method had advantages over other methods, because the parameters could be precisely measured and therefore were fully repeatable, and the exposure to the fusigenic stimulus was very short when compared to chemical and viral methods.

Tight membrane contact, a pre-requisite for fusion between two cells, was achieved by dielectrophoresis. Dielectrophoresis is a phenomenon whereby the field strengths on either side of a polarised neutral particle (for example, a cell) in a non-uniform field will be unequal. The result is a net force which acts on the particle, to move it towards the region of highest field intensity. A cell encounters an enhancement of local field divergence when approaching another cell, and will tend towards the higher field strength. As a result, cells in a non-uniform field produce chain-like aggregates (pearl chains), parallel to the field direction. "Pearl chains" are an essential element of cell fusion, since to initiate fusion close membrane contact must be established. The first step in the initiation of fusion is a reversible electrical breakdown in the zone of membrane contact between adjacent cells. When a cell membrane undergoes rapid polarization to a high voltage, an electrical breakdown of the membrane is observed,

coupled with dramatic, but reversible changes in conductance and permeability. Breakdown usually occurs at the lipid protein junction, or within the proteins, when a critical membrane thickness is reached (approximately 10-20% compression of the membrane locally to the high field intensity), and is the result of the electric compression forces changing more rapidly than the elastic forces restoring the membrane. As a result of electrical breakdown, pores are generated in the two opposed lipid bilayers of adjacent cells. Free lipid molecules tend to randomly accumulate in the pores and aggregate between the two membranes during "resealing" to form "bridges" between adjacent cells, which, in turn, leads to an open channel. The curvature of the channels and the high surface tension mean that the configuration is unstable, so that the process of fusion resulting in a spherical cell is energetically favoured. This electric field-induced fusion (electrofusion) technique has successfully been applied to all manner of cells and artificial vesicle systems (plant protoplasts and vacuoles; erythrocytes; lymphocytes and hybridomas; lipid vesicles).

Richter *et al* (1981) demonstrated that after using the technique of dielectrophoresis and electrofusion on "pearl chains" of sea urchin eggs, that the eggs retained the ability to be fertilized. The electrofusion technique was adapted by Kubiak and Tarkowski (1985) to fuse mammalian embryos cells, using 2-cell cleaving mouse embryos. In 2-cell mouse embryos with an intact zona, the natural contact between blastocysts is strong enough to permit cell fusion, without needing to perform dielectrophoresis. Kubiak and Tarkowski used a chamber for electrofusion which consisted of a petri-dish with a fixed and a variable electrode (platinum), arranged so that embryos were fused individually.

As well as mouse embryos the same method of electrofusion has been employed to fuse rat 2-cell embryos (Kurischko and Berg, 1986) and rabbit 2-cell embryos (Ozil and Modlinski, 1986) as well as mouse 4-cell embryos (Winkel and Nuccitelli,1989). Ozil and Modlinski modified the design of the electrofusion chamber, replacing the

point electrodes with parallel electrodes, so that several embryos could be fused at once. A similar chamber was used in the studies of Kaufman and Webb (1990) and in this study.

Up to 20% of tetraploid embryos produced by the suppression of second cleavage division may be 2n/4n mosaics, when using cytochalasin B. (Tarkowski *et al*, 1977). Identification of putative tetraploids in the past has relied on cytogenetic techniques performed on embryonic and extraembryonic tissues. This method can only be applied to dividing cells, which constitute a small proportion of the total number of cells in the conceptus, so that is difficult to say whether the sample is typical of the particular tissue studied, or of the embryo as a whole. The ideal solution to the problem would be a cellular marker with which it was possible to distinguish diploid and tetraploid cells in histological sections, thereby determining the exact location and distribution of tetraploid cells over the whole conceptus. This study employs the ß-globin transgenic cell marker described in Chapter 2.4, which may be detected by *in situ* hybridization.

By producing tetraploids from embryos carrying the transgene, tetraploid cells may be distinguished from diploid cells by the presence of hybridization signals present in the nucleus after performing *in situ* hybridization. For instance, if diploid embryos which were homozygous for the transgene (two spots per nuclei) underwent electrofusion, the resulting tetraploid embryos would have four copies of the transgene per cell, and thus four spots per nucleus. In this study, the technique of *in situ* hybridization was used to investigate tetraploid conceptuses produced by electrofusion. By determining whether, and what percentage of, fused embryos were mosaic it was possible to decide whether the process of electrofusion was a consistent enough technique to be used in the production of tetraploid↔diploid chimaeras. Fused embryos must be one hundred percent tetraploid to allow accurate identification of the fate of the tetraploid cell population in chimaeric conceptuses, without karyotyping each cell. The *in situ*

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hybridization technique facilitates estimation of the proportion of diploid:tetraploid cells in tetraploid conceptuses, as well as their distribution within the embryo.

3.2 MATERIALS AND METHODS

TETRAPLOID PRODUCTION

The tetraploid embryos were kindly produced by Dr. S.Webb (Dept. Anatomy, Edinburgh), using the technique of electrofusion described previously by Kaufman and Webb, (1990), which is discussed more fully in Chapter 5.2. BF_1 females were superovulated (see appendix II) and mated to CLO males which were homozygous for the ß-globin transgene (see appendix XI for explanation of the mouse strains).Two-cell stage embryos for electrofusion were flushed from the oviduct at $1^1/_2$ days of gestation (see appendix III). After electrofusion, the fused reconstituted "one-cell stage" embryos were transferred to the oviducts of BF_1 pseudopregnant females (Kaufman and Webb, 1990) on the first day of psuedopregnancy (see appendix I).

DIPLOID CONTROLS

Two series of diploid control conceptuses, homozygous and hemizygous for the ß-globin transgene, were kindly generated by Jean Flockhart in order to estimate the extent of retardation in the tetraploid embryos and to compare the percentages of labelled nuclei in the tetraploid embryos with those in homozygous diploid and hemizygous diploid embryos. CLO females mated to CLO males, resulted in diploid embryos with two copies of the ß-globin transgene (*Tg/Tg*). BF_1 females mated to CLO males produced hemizygous (single copy) diploid embryos (*Tg*/-). Eight cell embryos were flushed from the donor females, (see appendix II) and the embryos were transferred immediately to the uterus of pseudopregnant BF_1 females (see appendix III).

Table.3.1: Calculation of the nuclear diameter of tetraploid and diploid cells to determine the ratio of section thickness.

	Volume $(4/3)\pi r^3$	Radius r	Diameter 2r
Diploid (2n)	4.19	1	2.00
Tetraploid (4n)	8.38	1.26	2.52
Ratio $4n/2n$	2.00	1.26	1.26

Table 3.2: Measurements of nuclear length and breadth in various tissues of a $7^1/_2$ day old tetraploid embryo and $6^1/_2$ day old diploid embryos to assess nuclear diameter

Tissue					
Length (μm)	Breadth (μm)	Mean L+B	Length (μm)	Breadth (μm)	Mean L+B
Tetraploid			Diploid		
Embryo					
12.1	8.6	10.34	10.8	6.5	8.65
15.5	10.3	12.93	8.6	4.3	6.49
15.5	12.1	13.79	6.5	5.4	5.95
12.1	10.3	11.21	8.6	6.5	7.57
13.8	10.3	12.07	9.7	8.7	9.19
12.1	7.8	9.91	7.6	6.5	7.03
13.8	12.9	13.36	6.5	4.3	5.41
10.3	8.6	9.48	7.6	5.4	6.49
Parietal Endoderm					
12.1	8.6	10.34	8.6	2.7	5.68
15.5	10.3	12.93	7.6	4.3	5.95
10.3	6.9	8.62	8.6	4.3	6.49
10.3	6.0	8.19	7.6	7.0	7.30
Ectoplacental Cone					
17.2	17.2	17.24	8.6	7.6	8.11
10.3	7.8	9.05	6.5	5.4	5.95
10.3	6.9	8.62	8.6	6.5	7.57
10.3	6.9	8.62	9.7	8.7	9.19
Trophoblast					
13.8	10.3	12.07	8.6	7.8	8.11
12.1	11.2	11.64	7.6	7.8	7.57
10.3	10.3	10.34	9.7	4.3	7.03
15.5	12.1	13.79	8.6	7.8	8.11

Table 3.3:Comparison of mean nuclear diameter of tetraploid and diploid cells.

Tissue	No. of nuclei	Mean (±SE) Nuclear Diameter (μm)		MeanRatio 4n/2n
		Tetraploid	Diploid	
Embryo	8	11.64 ±0.58	7.10 ±0.46	1.64
Parietal endoderm	4	10.02 ±1.08	6.35 ±0.36	1.58
Ectoplacental cone	4	10.88 ±2.12	7.70 ±0.68	1.41
Trophoblast	4	11.96 ±0.71	7.70 ±0.26	1.55
Total Mean Ratio (all tissues)				**1.56**

HISTOLOGY

Putative tetraploid conceptuses were recovered at $7^1/_2$, $8^1/_2$, $9^1/_2$ and $10^1/_2$ days of gestation, diploids at $6^1/_2$, $7^1/_2$ and $8^1/_2$ days and fixed in 3:1 ethanol:acetic acid fixative (see appendix VII). The samples were then processed for wax histology. Sections of the tetraploid conceptuses were cut at 7μm and to compensate for the smaller size of the diploid cells, the diploid conceptuses were sectioned at 5μm, so that a similar proportion of nucleus was present (see Chapter 3.3).

In situ HYBRIDIZATION

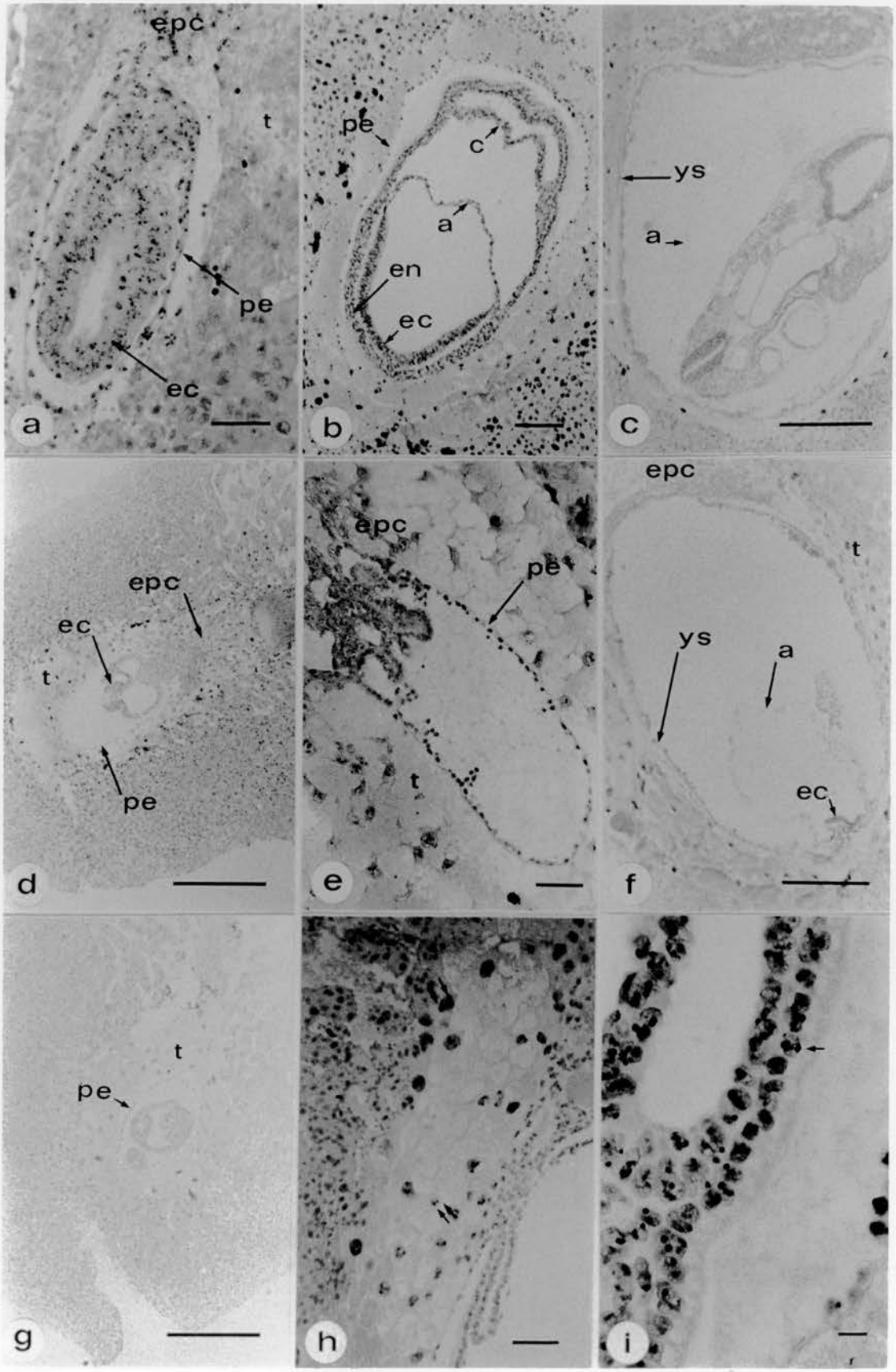
In situ hybridization was performed as described in Chapter 2.4, using the DNA probe, pMßð2 labelled with digoxygenin (see appendix VIII).

3.3 RESULTS

SECTION THICKNESS

As discussed in Chapter 2.4 the choice of section thickness is an important factor when performing *in situ* hybridization. Tetraploid sections were cut at 7μm, but since the diploid nuclei are expected to be smaller than tetraploid nuclei, thinner sections were used to try and ensure that a similar proportion of the nucleus was present in the sections. The ratio of nuclear diameters (4n:2n) was estimated in two ways. Firstly, (Table 3.1) assuming that the tetraploid:diploid nuclear volume ratio is 2:1 (Henery and Kaufman, 1992), the corresponding ratio for nuclear diameters can be calculated as 1.26:1 (Epstein, 1986), given that the volume of a sphere is $(^4/_3)\pi r^3$. To ensure that a comparable proportion of the nucleus was included in histological sections, the section thickness for tetraploid samples should be 1.26 x greater than for diploid tissue. For tetraploid samples cut at 7μm, the appropriate thickness for diploid sections would be 5.56μm. A second calculation was made, based on observed nuclear diameters, in matched material. The nuclear length and breadth was measured in 8 areas of the embryo, 4 areas of parietal endoderm, 4 areas of ectoplacental cone and 4

Fig 3.1 Histological sections of diploid (*a,b,c & i*) and tetraploid (*d,e,f,g & h*) mouse conceptuses, after *in situ* hybridization to detect the transgenic ß-globin sequence (*Tg*), showing poor embryonic development of tetraploids. Bar is 10μm in *i*, 50μm in *a*, 100μm in *b, e & h* and 500μm in *c, d, f & g*. *(a)* Normal $6^1/_2$ day homozygous, *Tg/Tg* diploid; *(b)* normal $7^1/_2$ day heterozygous, *Tg/+* diploid; *(c)* normal $8^1/_2$ day homozygous, *Tg/Tg* diploid. *(d-g)* "Hemizygous", *Tg/Tg /+/+* tetraploid conceptuses at *(d)* $7^1/_2$ days, *(e)* $8^1/_2$ days, *(f)* $9^1/_2$ days and *(g)* $10^1/_2$ days. *(h)* Part of a $10^1/_2$ day "hemizygous", *Tg/Tg /+/+* tetraploid showing trophoblast giant cells with two hybridization signals (arrows). *(i)* High power of part of normal $7^1/_2$ day heterozygous, *Tg/+* diploid conceptus (*b*), showing one hybridization signal in most nuclei. Abbreviations: a, amnion; c, chorion; ec, embryonic ectoderm; en, embryonic endoderm with subjacent mesoderm; epc, ectoplacental cone; pe, parietal endoderm; t, trophoblast; ys, visceral yolk sac.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areas of trophoblast of a $7^1/_2$ day tetraploid conceptus and a $6^1/_2$ day hemizygous diploid conceptus (Table 3.2). The average of the two values was calculated for each cell to estimate the nuclear diameter and the mean for each tissue calculated. The mean ratio between tetraploid and diploid nuclear diameters was calculated for each tissue along with the ratio for all tissues (Table 3.3). The mean ratio of 1.56, was used to calculate a section thickness for the diploid embryos of 4.48μm (equivalent to 7μm for the tetraploid). The actual section thickness used for the diploid embryos was 5μm, which is close to both the theoretical (5.56μm) and calculated (4.48μm) values.

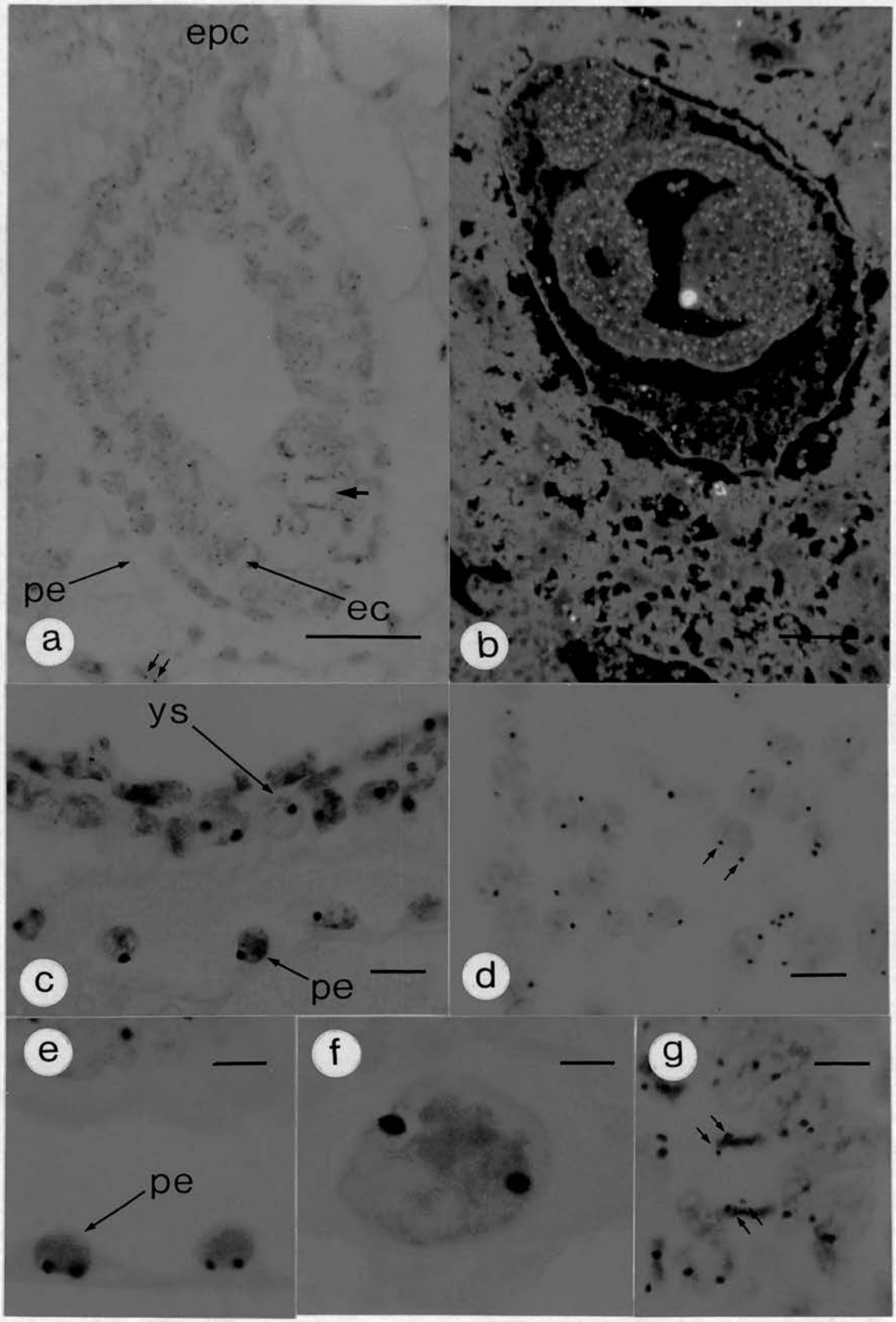
MORPHOLOGY

The development of the tetraploid embryos was retarded with respect to the diploid control conceptuses which agrees with previous studies of tetraploid development (Snow, 1975, Tarkowski *et al*, 1977). Figs. 3.1 *a-c* ($6^1/_2$, $7^1/_2$ and $8^1/_2$ day old respectively) show histological sections of diploid embryos displaying increasing complexity with age. By comparison, Figs. 3.1*d-g* ($7^1/_2$, $8^1/_2$, $9^1/_2$ and $10^1/_2$ day old respectively) illustrate typical morphology of tetraploid conceptuses. Extraembryonic membranes survived better than embryonic tissues which were much reduced and disorganised. The parietal endoderm, yolk sac and trophoblast were usually visible (Fig. 3.1*g*), and were the least affected structures in the tetraploid conceptuses. This agrees with the observations of Tarkowski *et al* (1977), who also reported that the major problem during tetraploid development was the lack of mesoderm, and that mesoderm present was limited to the fetal membranes. In some cases the tetraploid conceptuses were surrounded by maternal blood outside the parietal endoderm.

SCORING THE *in situ* HYBRIDIZATION SIGNAL

In situ hybridization was carried out on all slides which had sections containing the embryo. Sections showing labelled nuclei after *in situ* hybridization, (indicating the presence of embryonic tissue) were noted and a section near the middle of the range of

Fig 3.2 *(a & b)* "Hemizygous", *Tg/Tg* /+/+ tetraploid mouse conceptuses, after *in situ* hybridization to detect the transgenic ß-globin sequence *(Tg)* in histological sections, at *(a)* $7^1/_2$ days and *(b)* $10^1/_2$ days (dark field photograph of conceptus shown in Fig 1*g*). Each conceptus has nuclei with two hybridization signals, including some trophoblast giant cells (small arrows). A mitotic figure indicated in *(a)* (heavy arrow) is shown in more detail in *(g)*. *(c & d)* High power photographs of nuclei from normal diploid embryos after *in situ* hybridization. *(c)* Nuclei with one hybridization signal in the parietal endoderm of a $7^1/_2$ day heterozygous, *Tg*/+ diploid conceptus; *(d)* Neurectoderm of an $8^1/_2$ day homozygous, *Tg/Tg* diploid conceptus with some nuclei with two hybridization signals. *(e-g)* High power photographs of nuclei from "hemizygous", *Tg/Tg* /+/+ tetraploid embryos after *in situ* hybridization. *(e)* Parietal endoderm from $9^1/_2$ day tetraploid and *(f)* trophoblast giant cell $10^1/_2$ day tetraploid, each with two signals per nucleus. *(g)* "Hemizygous" tetraploid mitotic figure from $7^1/_2$ day conceptus, showing segregation of two pairs of labelled chromosomes (arrows indicate the two hybridization signals moving to each pole). Bar is 10µm in *c, d, e, f* & *g*, 50µm in *a* and 100µm in *b*. Abbreviations as in Fig 1.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these sections was chosen for scoring (Fig. 3.2). The number of hybridization signals visible in the nuclei of different diploid tissues was counted. Diploids hemizygous for the transgene had one spot per nucleus and homozygotes two spots. Tetraploid embryos produced from diploid hemizygous (*Tg/-)* embryos should have two copies of the transgene per nucleus (*Tg/Tg/-/-*) (Fig. 3.2*a*), as the homozygous diploid (*Tg/Tg*) embryos do (Fig. 3.2*d*). The number of spots per nucleus counted in the tetraploid embryos were compared with those counted in control hemizygous and homozygous diploid embryos. A uniformly "hemizygous" tetraploid tissue (*Tg/Tg/-/-*) would be expected to have a similar proportion of double-spot nuclei as the homozygous (*Tg/Tg*) controls. A mosaic tissue which was a mixture of *Tg/Tg/-/-* and *Tg/-* cells would have a lower percentage of nuclei with 2 spots.

The tissues scored were: embryo, parietal endoderm, ectoplacental cone and trophoblast (Figs. 3.1 *a-g*.) All four of these structures were easy to distinguish in early stage conceptuses. Embryonic and extraembryonic tissues were included in the scoring system to ensure that if 4n/2n mosaicism was confined to one developmental lineage, it would still be detected. Apart from the parietal endoderm where the whole structure was scored, areas of tissue to be counted were delineated in a Leitz Diaplan microscope using an eye-piece with a 10x10 grid. Using a x25 objective and x12.5 (20mm) eye-piece, a single square, (corresponding to 50μm x 50μm on the slide) was selected and each of the nuclei contained in that area was scored as: 0 signal, 1 signal, 2 signals or unscoreable (unscoreable cells were those which had overlapping nuclei). One area each of trophoblast tissue and ectoplacental cone tissue was scored for each embryo, and two areas of embryonic tissue.

The mean and the standard error for the percentage of each of the categories in the scoring system, 0 signal, 1 signal, 2 signals or unscoreable, were calculated for each age of embryo, for both the tetraploid and diploid series. The number of embryos and

Table 3.4: The proportion and percentage of hybridization signals in various tissues of "hemizygous" tetraploid embryos of different developmental ages, sectioned at 7μm and detected by *in situ* hybridization.

Tissue	Age (days)	No. of Embryos scored	Total Nuclei Scored	Nuclei with 0 signal	Nuclei with 1 signal	Nuclei with 2 signals	% positive nuclei with 2 signals
Embryo							
	7 1/2	3	86	23	23	40	63.5
	8 1/2	4	101	19	34	48	58.5
	9 1/2	4	133	29	56	48	46.1
	10 1/2	3	90	26	36	28	43.7
Parietal endoderm							
	7 1/2	3	97	18	32	47	59.5
	8 1/2	4	249	53	74	122	62.2
	9 1/2	4	482	107	145	230	61.3
	10 1/2	3	163	41	70	52	42.6
Ectoplacental cone							
	7 1/2	3	46	11	11	24	68.6
	8 1/2	2	25	3	11	11	50.0
	9 1/2	2	24	5	8	11	57.9
	10 1/2	2	24	4	9	11	55.0
Yolk Sac (whole)							
	9 1/2	2	502	111	180	211	54.0
Yolk Sac Endoderm*							
	9 1/2	1	24	2	14	8	36.4
Yolk Sac Mesoderm*							
	9 1/2	1	18	2	5	11	68.7
Trophoblast							
	7 1/2	1	64	**	33	31	48.4
	8 1/2	1	9	**	5	4	44.4

* From same embryo

** All trophoblast cells scored were positive (hybridization signal distinguished them from maternal decidua cells)

Table 3.5: The proportion and percentage of hybridization signals in various tissues of diploid homozygous embryos of different ages, sectioned at 5μm and detected by *in situ* hybridization.

Tissue	Age (days)	No. of Embryos scored	Total Nuclei Scored	Nuclei with 0 signal	Nuclei with 1 signal	Nuclei with 2 signals	% positive nuclei with 2 signals
Embryo							
	6 1/2	3	123	21	46	56	54.9
	7 1/2	2	98	21	31	46	59.7
	8 1/2	3	142	25	54	63	53.8
Parietal endoderm							
	6 1/2	2	65	14	28	23	45.1
	7 1/2	3	193	55	67	71	51.4
	8 1/2	3	327	70	138	119	46.3
Ectoplacental Cone							
	6 1/2	3	63	14	21	28	57.1
	7 1/2	2	43	12	17	14	45.2
	8 1/2	3	64	16	20	28	58.3
Amnion							
	8 1/2	1	78	22	31	25	44.6

Table 3.6: The proportion and percentage of hybridization signals in various tissues of diploid hemizygous embryos of different ages, sectioned at 5μm and detected by *in situ* hybridization.

Tissue	Age (days)	No. of Embryos scored	Total Nuclei Scored	Nuclei with 0 signal	Nuclei with 1 signal	Nuclei with 2 signals	% positive nuclei with 2 signals
Embryo							
	6 1/2	3	121	46	72	3	4.0
	7 1/2	3	124	49	72	3	4.0
	8 1/2	1	50	10	38	2	5.0
Parietal endoderm							
	6 1/2	3	91	39	51	1	1.9
	7 1/2	3	167	53	111	3	2.6
	8 1/2	1	154	57	92	5	5.1
Ectoplacental cone							
	6 1/2	3	57	27	29	1	3.3
	7 1/2	2	39	17	22	0	0
	8 1/2	1	26	11	14	1	6.7
Yolk Sac (whole)							
	8 1/2	1	45	11	34	0	0
Trophoblast							
	7 1/2	1	16	6	9	1	10

Table 3.7: Mean frequencies of nuclei with 0 or 2 hybridization signals in the embryo, parietal endoderm and ectoplacental cone.

Tissue	Expected Genotype					
	2n hemizygote proportion	%	2n homozygote proportion	%	4n "hemizygote" proportion	%
Overall proportion of nuclei with no signal:						
Embryo	105/295	35.6	67/363	18.5	97/410	23.7
P.Endoderm	149/412	36.2	139/585	23.8	219/991	22.1
E.P.Cone	55/122	45.1	42/170	24.7	23/119	19.3
Overall proportion of positive nuclei with 2 signals:						
Embryo	8/190	4.2	165/296	55.7	164/313	52.4
P.Endoderm	9/263	3.4	213/446	47.8	451/772	58.4
E.P.Cone	2/67	3.0	70/128	54.7	57/96	59.4

P.Endoderm: parietal endoderm
E.P.Cone: ectoplacental cone

Table 3.8: Statistical significance (χ^2 test*) of the differences, in frequency of hybridization signals per nucleus, between tetraploid and both groups of diploid conceptuses.

	No Signal		2 Signals	
	χ^2	P	χ^2	P
2n Hemizygous versus 4n "hemizygous"				
Embryo	11.95	*P=0.0005*	119.86	*P<0.0001*
Parietal endoderm	29.76	*P<0.0001*	238.09	*P<0.0001*
Ectoplacental cone	18.25	*P<0.0001*	51.92	*P<0.0001*
2n Homozygous versus 4n "hemizygous"				
Embryo	3.12	P=0.0775	0.69	P=0.4075
Parietal endoderm	0.58	P=0.4468	12.96	*P=0.0003*
Ectoplacental cone	1.16	P=0.2812	0.49	P=0.4835

* Yates correction was applied when expected frequencies fell below 10. Differences were considered significant if P<0.05 (shown in italics).

Table 3.9: Statistical significance (χ^2 test*) of the differences, in frequency of hybridization signals per nucleus, between tissues in tetraploid conceptuses.

Comparison	Proportion of nuclei with no signal		Proportion of nuclei with 2 signals	
	χ^2	P	χ^2	P
Embryo v Parietal endoderm	0.40	0.5251	3.29	0.0697
Embryo v Ectoplacental cone	0.99	0.3206	1.44	0.2300
Parietal endoderm v Ectoplacental cone	0.48	0.4891	0.03	0.8578

* Yates correction was applied when expected frequencies fell below 10.
No differences were considered statistically significant (none with $P<0.05$).

the total number of nuclei scored for each age and tissue can be seen in tables 3.4, 3.5 and 3.6 (tetraploid, diploid homozygous and diploid hemizygous respectively). The proportion of nuclei with no signal gives an indication of the percentage of false negatives which occurred due to failure of the *in situ* technique (includes hybridization failure and absence of target sequences from sections). Also calculated was the proportion of positive nuclei with two signals. The total proportion of nuclei with no signal and positive nuclei with two signals for each tissue type, disregarding age, is shown in Table 3.7. The results were tested statistically using a χ^2 test (Table 3.8). The data for the "hemizygous" tetraploid embryos was tested statistically against both the hemizygous and homozygous diploid values. Tables 3.7 and 3.8 show that the "hemizygous" tetraploid group had significantly fewer nuclei with no signal and significantly more nuclei with two signals than the hemizygous diploid group. Comparison of "hemizygous" tetraploid and homozygous diploid groups revealed no significant difference for the embryo or ectoplacental cone. Although a significant difference in the frequency of nuclei with two signals was detected in the parietal endoderm this does not provide evidence for mosaicism in the "hemizygous" tetraploid group because the frequency of nuclei with two signals was higher in the 4n than the homozygous diploid parietal endoderms. To determine whether this difference was due to non-homogeneity in the sample group, a second test was undertaken which accounted for greater variability between embryos than within embryos. Weighted means and S.E.M's were calculated for the diploid homozygote and tetraploid "hemizygote" parietal endoderm groups (47.78±0.649; 58.42±0.703 respectively), and a Student's "t" test was carried out ($t=2.947$; $P<0.01$). The difference between the diploid homozygote and tetraploid "hemizygote" parietal endoderms remained significant, but was less so with this test. χ^2 tests were also carried out between the different tissues scored in tetraploid embryos (Table 3.9). There was no significant difference between the different tissue types tested. This supports the conclusion that the discrepancy in the parietal endoderm χ^2 values is not biologically significant.

3.4 DISCUSSION

The development of the tetraploid embryos generated by this particular combination of strains was poorer than that previously reported by Kaufman and Webb (1990). Viable embryos have been recorded to term (Snow, 1975) and 16 days of gestation (equivalent in developmental stage to normal embryos of about 14-14$^1/_2$ days p.c., Kaufman, 1991a). There are two possible explanations for this observation; either the development of tetraploid embryos varies according to the strain combination (genetic background) or that the presence of the transgene is somehow interacting to hamper development in the tetraploid embryo in a way not observed in diploids generated from the same strain. The former explanation would seem more plausible, since strain dependent effects on development have been reported in several different instances for both tetraploidy (Tarkowski *et al*, 1977; Dyban and Baranov, 1987; Webb *et al*, 1992) and triploidy (Wróblewska, 1971; Niemierko, 1981). In the series of tetraploid mouse embryos described by Snow (1975), the embryonic ectoderm was commonly reduced and disorganised, the cells being loosely arranged and fewer in number than diploids of the same age. Tarkowski *et al*, (1977) noted that growth of tetraploid conceptuses was generally slower than in normal diploid embryos. By 8$^1/_2$ days of gestation the embryonic portion of the conceptus appeared underdeveloped with either an absence of, or vestigial embryonic structures. The type of morphological changes observed in tetraploid conceptuses have also been seen in embryos with other chromosome abnormalities (Dyban and Baranov, 1987). For instance, the triploidy syndrome described by Wróblewska (1971) involved a general retardation of growth, but was also strain dependent, since in appropriate strain combinations, limb bud stage diandric and digynic triploid embryos are observed (Kaufman, 1991b), and more recently Bos-Mikich and Whittingham (1992) reported triploid mouse fetus at 14 days of gestation (equivalent to 13 days of gestation). By around 8-9 days of gestation the embryonic region of the triploidy syndrome conceptuses described by Wróblewska was small in

comparison to the extraembryonic region. The primary germ layers were less differentiated, with fewer layers and rounded cells. These observations closely resemble morphology seen in tetraploid embryos, although the tetraploid phenotype manifests earlier and often results in no more than a trophoblastic vesicle (Graham, 1971, Snow, 1976). Tetraploid embryos have been recovered at between 10 days of gestation (Tarkowski, 1977), which resembles more closely the conceptuses collected in this study, and term reported by Snow (1975). However, recent attempts to repeat the success achieved by Snow, have resulted in embryos no older than 16 days of gestation (Kaufman, 1991a). There may be two explanations for these differences. Firstly, that the differences in development are due to the strain combinations used, or that the most advanced of Snow's tetraploids were perhaps 4n/2n mosaics and that the cytogenetic analysis failed to identify them as such. Even a small proportion of diploid cells in a mosaic embryo may have been sufficient to partially rescue the embryo and allow development proceed further than in a solely tetraploid embryo.

A low incidence of nuclei with two positive signals was observed in all tissues of the hemizygous diploid (*Tg*/-) conceptuses (Table 3.5). There are two possible explanations for this observation. Firstly, that the cells are genuinely tetraploid, as observed, for example in the visceral endoderm and trophoblast (Zybina, 1970; Ilgren, 1980). Alternatively, the apparent polyploid cells observed may be technical artefacts, due to overlapping cells, despite the fact that the sections were cut at 5μm to compensate for the smaller nuclear diameter of the diploid cells. The latter seems less likely since closely packed cells or cells which did not have obvious nuclear borders were counted as unscoreable.

The incidence of 2 signals per nucleus in homozygous diploid (and "hemizygous" tetraploid) conceptuses is only ~50%, when, theoretically, the expected value is 100%. This is most likely to be an artefact attributable to the use of histological

sections, because some target sequences will be excluded from part of the sectioned nuclei particularly in thin sections.

Tarkowski *et al* (1977) found that a proportion, around 20%, of the tetraploid embryos he produced, using cytochalasin B, were 4n/2n mosaics as detected cytogenetically. The ability to detect mosaicism by *in situ* hybridization is reduced by the failure to detect both hybridization signals in about 50% of the nuclei expected to have two copies of the transgene. Nevertheless it should have been possible to recognise the presence of diploid cells in the tetraploid embryos, if they comprised at least 15% of the total nuclei scored, as explained below.

If the proportion of all nuclei scored that had one hybridization signal in the embryonic region of the 4n group was 177/410 (43.2%), instead of 149/410 (36.3%; nuclei with one signal/total nuclei scored, Table 3.4), then this would have been significantly higher than the corresponding proportion for the homozygous diploid embryos; 131/363; 36.1% (χ^2 =4.03; P=0.045). This higher proportion of nuclei with one signal could have been accounted for by 11.1% of diploid nuclei in the "tetraploid" embryos; as calculated by

$$\left[1-\left(\frac{\text{0+2 spots in 4n}}{\text{All nuclei}}\right)/\left(\frac{\text{0+2 spots in 2n homozygous}}{\text{All nuclei}}\right)\right]\times 100$$

ie.

$$\left[1-\left(\frac{233}{410}\right)/\left(\frac{232}{363}\right)\right]\times 100$$

(from Lyon,1970)

Therefore it would have been possible to detect the presence of diploid cells in the tetraploid embryos if they composed at least 11.1% of the total nuclei scored.

A similar calculation, ignoring the nuclei with no hybridization signals, predicted that 2n/4n mosaicism would be detected if at least 14.6% of the nuclei were diploid.

The frequency of nuclei with no hybridization signal was higher in the hemizygous diploid series. This was expected since there is a greater chance of failing to include the target sequence in a section when there is only one target per nucleus, compared to two sequences in both the homozygous diploid and "hemizygous" tetraploid series. The frequency of positive nuclei with two signals in "hemizygous" 4n (*Tg/Tg/-/-*) conceptuses was not significantly lower than that observed in the homozygous diploid (*Tg/Tg*) conceptuses. This implies that the tissues in the tetraploid conceptuses are uniformly 4n, and consequently that there is no evidence that the electrofusion technique results in 4n/2n mosaicism.

CHAPTER 4

DIPLOID↔DIPLOID CONTROL CHIMAERAS

4.1 INTRODUCTION

Before embarking upon the production of tetraploid↔diploid chimaeras, several control experiments were carried out which involved generating diploid↔diploid chimaeras. Firstly, chimaeras were made by aggregating two types of F_2 embryos, AF_2 and BF_2 , that differed at the *Gpi-1s* locus (*Gpi-1s*^{a}/*Gpi-1s*^{a} and Gpi-1sb/*Gpi-1s*^{b} respectively) and the proportions of cells derived from each embryo were estimated by quantitative electrophoresis of glucose phosphate isomerase (GPI). The AF_2↔BF_2 chimaeras were transferred to AF_1 foster mothers, which meant that any maternal contamination in tissues of the conceptuses would remain undetected. To control for this contamination, a series of BF_2 embryos were transferred directly to AF_1 females.

4.2 MATERIALS AND METHODS

CHIMAERA PRODUCTION

AF_1 and BF_1 (see appendix XI) female mice were superovulated (see appendix II) and mated to males of the same F_1 strain. (AF_1 x AF_1)F_2 and (BF_1 x BF_1)F_2 embryos, abbreviated to AF_2 and BF_2 respectively, were collected $2^1/_2$ days *post coitum* (*p.c.*), at the 8-cell stage (see appendix II). After collection the zonae pellucidae were removed in acidic Tyrode's solution at pH 2.5 (Nicholson *et al*, 1975) and the denuded embryos were aggregated into pairs (see appendix IV). Aggregated pairs consisting of one AF_2 and one BF_2 embryo were cultured overnight and transferred to AF_1 pseudopregnant females (see appendix IV).

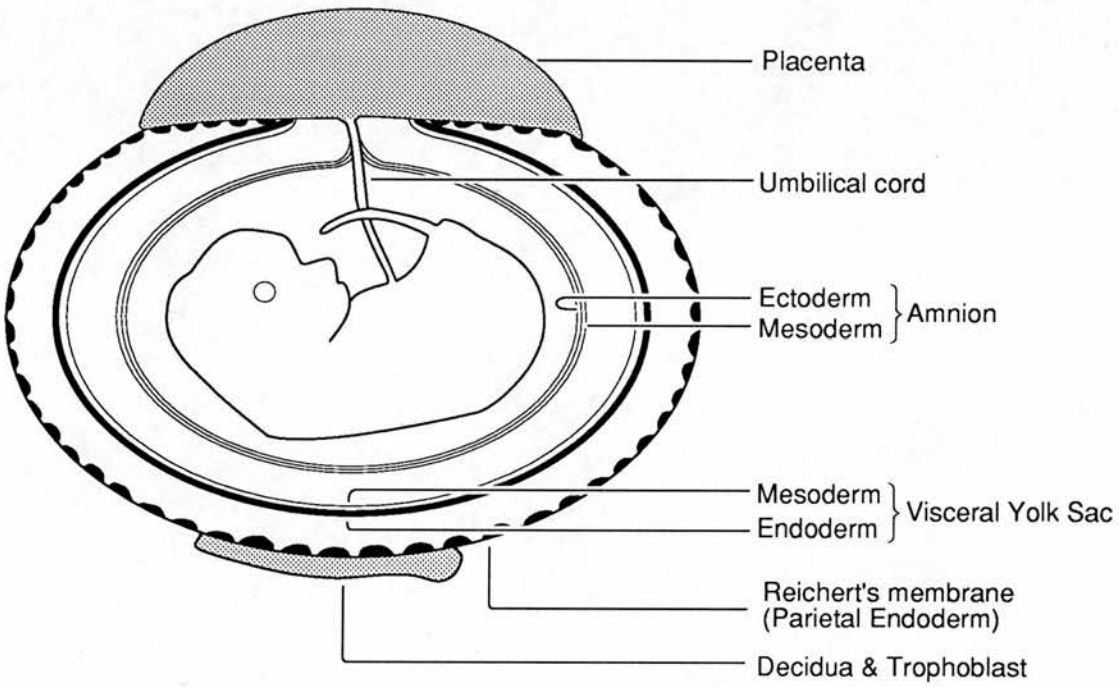


Fig.4.1 Diagram of $12^1/_2$ day mouse conceptus (re-drawn after Papaioannou and West, 1981). The different tissues are shaded according to their developmental lineage. The fetus, amnion and yolk sac mesoderm (unshaded) are derived from the primitive ectoderm lineage; the yolk sac endoderm and parietal endoderm of Reichert's membrane (shaded black) are derived from the primitive endoderm lineage; the placenta and band of tissue on the outer surface of Reichert's membrane (shaded grey) are mainly a mixture of trophoblast (trophectoderm lineage) and maternal decidual tissue, although the placenta has minor contributions from other lineages.

TRANSFER CONTROL EMBRYOS

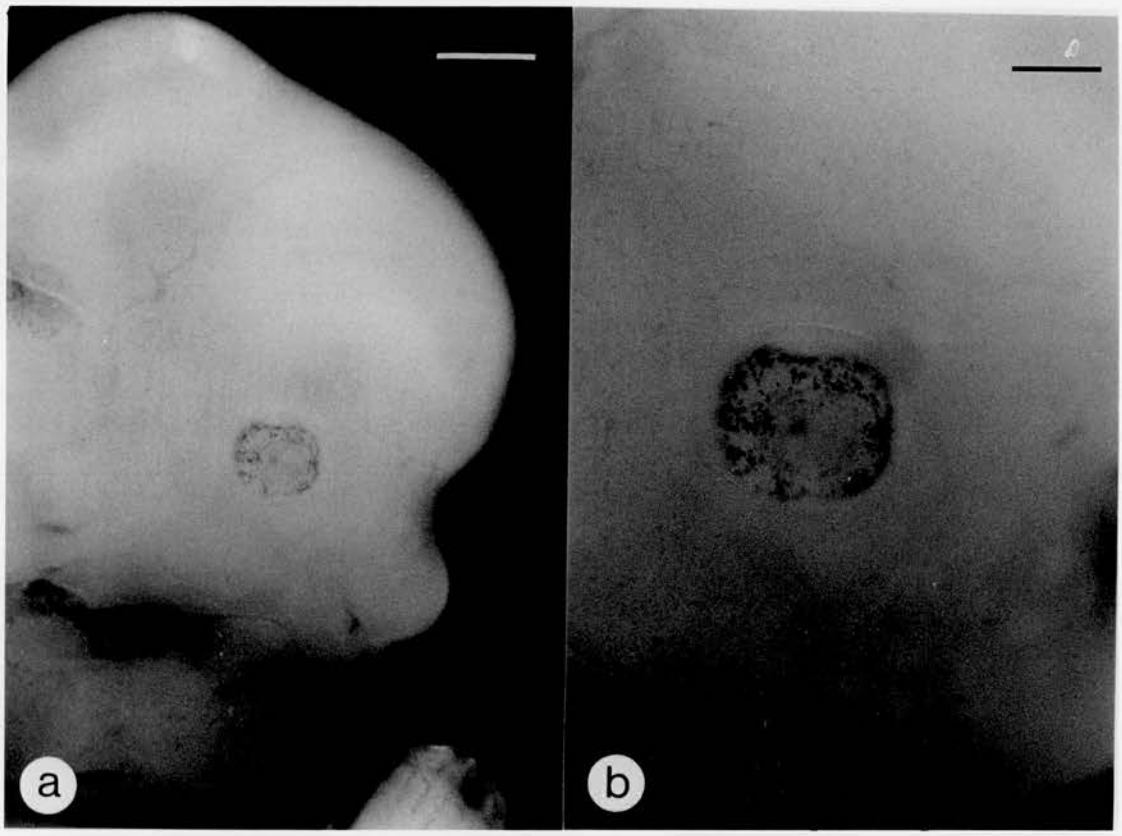
AF_1 pseudopregnant females were used as recipients for the $AF_2 \leftrightarrow BF_2$ chimaeras, so that any maternal contamination would result in an elevated proportion of GPI-1A in tissues which were contaminated. The extent of maternal contamination was determined by assessing the GPI-1A content of a series of control embryo transfers which was generated, dissected and assayed by Jean Flockhart and John West. BF_1 embryos were collected at $3^1/_2$ days *p.c.* and surgically transferred to AF_1 pseudopregnant females (see appendix IV).

EMBRYO DISSECTIONS

For both the diploid↔diploid chimaeras and the transfer control embryos, the recipient females were sacrificed at $12^1/_2$ days *p.c.*, and the resulting conceptuses were analysed in the same manner. The conceptuses were dissected, as described by Papaioannou and West (1981) (see appendix V).The dissected tissues; placenta, trophoblast/decidual tissue, Reichert's membrane, yolk sac endoderm, yolk sac mesoderm, amnion, fetus and umbilical cord (Fig. 4.1) were prepared for enzyme electrophoresis (see appendix V). A rough estimate of the contribution of the BF_2 (pigmented) component in the chimaeras was made by observing the distribution of pigmented cells in the retinal epithelium of the eye (Fig. 4.2*a* & *b*).

The homogenized tissues were subjected to gel electrophoresis as described in Chapter 2.3 (see appendix VI) to assay for the enzyme GPI. Scanning densitometry was used to determine the proportions of each alloenzyme type, GPI-1A and GPI-1B in the tissue samples. The proportion of GPI-1A:GPI-1B was used to estimate the cellular contribution of each strain type to the resulting chimaera.

Fig 4.2 *(a)* and *(b)* Low and high power images of the head of a $12^1/_2$ day $AF_1 \leftrightarrow BF_1$ (XE series) chimaeric fetus, showing the pattern of mixing of pigmented cells of the BF_1 genotype with non-pigmented of the AF_1 genotype in the pigmented retinal epithelium (bar is 1000μm in *(a)* and 500μm in *(b)*).


a
b

4.3 RESULTS

ANALYSIS OF TRANSFER CONTROLS

Thirty four conceptuses were recovered from the transfer of BF_1 embryos to AF1 pseudopregnant females. The percentage of maternally derived GPI-1A activity in various part of the conceptuses is shown in Table 4.1. The GPI analysis revealed that while the fetus, amnion, yolk sac mesoderm and yolk sac endoderm showed no maternal contamination, the parietal endoderm (Reichert's membrane), decidua/trophoblast and placenta displayed a significant proportion of GPI-1A. The umbilical cord had no maternal contamination, but it was not included in the analysis. Fourteen of 33 of the parietal endoderm samples were contaminated with maternally derived GPI-1A, but the overall mean contamination was only 3.31%. This low level was unlikely to affect the analysis, and so was not taken into account when analysing parietal endoderm samples. The decidua/trophoblast samples were predominantly GPI-1A, and 5/34 samples were 100% GPI-1A (presumably entirely decidua). The contamination was too great and too variable to allow for an accurate correction factor, so the trophoblast was omitted from this chimaera study. The placenta also had a high proportion of maternal GPI-1A enzyme (mean value 45.3%) presumably because the outer layer of the placenta is maternal in origin and because of the presence of maternal blood. However, the proportion of maternally derived GPI-1A was much less variable than the trophoblast, with a coefficient of variation of only 15.48. One placenta showed a much lower percentage of GPI-1A than average (11.5% compared to 45.3%). This placenta was also the lightest, (0.06g compared to 0.09g), and the fetus/placenta ratio was the highest at 2.00, when the mean was 1.29 (range of 0.80-1.67). This placenta was excluded from the calculations, since the low weight and low proportion of maternally derived GPI-1A suggest that the outer maternal layer of the placenta was lost during dissection. The mean proportion of GPI-1A in the remaining

Table 4.1: Proportion of GPI-1A (maternally-derived enzyme) in various tissues of 34 $12^1/_2$ day conceptuses produced by transferring homozygous *Gpi-1s*^{b}/*Gpi-1s*^{b} preimplantation embryos into homozygous *Gpi-1s*^{a}/*Gpi-1s*^{a} pseudopregnant recipient females.

Tissue	Proportion of conceptuses with GPI-1A	% GPI-1A (maternal contamination)		
		Mean±S.E.M.	Coefficient of variation (%)	Range
Fetus	0/34	0		
Amnion	0/34	0		
Yolk Sac Mesoderm	0/34	0		
Umbilical cord	0/34	0		
Yolk Sac Endoderm	0/34	0		
Parietal Endoderm	14/33	3.3 ± 0.81	140.13	0-19
Decidua/Trophoblast	34/34	74.6 ± 2.66	20.83	44-100
Placenta	34/34	44.3 ± 1.55	20.35	12-64
Placenta*	33/33	45.3 ± 1.22	15.48	32-64

* Second row of placenta data excludes one placenta with only 11.5% GPI-1A. This placenta was abnormally light and it is likely that most of the outer maternal layer was lost during dissection.

Table 4.2: % GPI-1A in tissues of $12^1/_2$ day chimaeric conceptuses, ranked by %GPI-1A in the fetus.

Chimaera Ref.	% GPI-1A							
	F	A	Y.S. meso	Y.S. endo	P. endo	Placenta	Placenta corr-1*	Placenta corr-2*
XE-21	100	100	100	41	16	46	2	2
XE-17	100	100	100	35	34	100	100	100
XE-6	100	100	100	52	77	100	100	100
XE-35	95	88	96	45	49	94	89	89
XE-16	91	88	94	92	100	100	100	100
XE-24	90	91	93	43	93	94	90	90
XE-33	88	81	71	52	46	90	82	82
XE-27	87	68	87	62	100	89	79	79
XE-19	85	85	94	47	100	54	16	16
XE-11	85	84	76	99	100	98	96	96
XE-13	73	81	72	23	85	98	96	96
XE-14	72	48	59	87	89	95	91	91
XE-8	72	78	72	37	39	99	98	98
XE-23	72	55	64	26	51	46	1	1
XE-28	71	85	64	54	79	81	66	66
XE-32	69	67	58	15	35	45	0	0
XE-12	68	77	78	59	42	56	19	19
XE-22	64	80	65	48	48	91	83	83
XE-29	63	61	49	21	58	48	5	5
XE-31	55	50	59	44	33	65	36	36
XE-26	51	48	45	43	61	22	-42	0
XE-9	47	47	50	68	53	58	24	24
XE-37	43	50	53	16	65	50	9	9
XE-34	41	43	45	43	37	50	9	9
XE-5	30	25	29	17	36	44	-2	0
XE-15	28	32	35	31	46	68	42	42
XE-2	26	43	22	43	93	45	0	0
XE-20	22	28	24	56	71	41	-7	0
Mean	67.41	67.24	66.18	46.36	61.96	70.18	45.77	47.61
S.E.M.	4.51	4.30	4.48	4.07	4.78	4.66	8.46	7.93
S.D.	23.89	22.74	23.72	21.52	25.31	24.63	44.79	41.97
N	28	28	28	28	28	28	28	28
Coeff Var.	35.44	33.82	35.84	46.42	40.85	35.11	97.85	88.15

Abbreviations: F, fetus; A, amnion; Y.S. Meso, yolk sac mesoderm; Y.S. Endo, yolk sac endoderm; P.Endo, parietal endoderm.

* Placental value was corrected to allow for 45% of maternal GPI-1 in the placenta.
Placenta corr-1: %GPI-1A = [(observed %GPI-1A - 45) x 100] / (100 - 45)
Placenta corr-2: the negative values are treated as 0.

33 placentas was approximately 45% (Table 4.1), which was the figure used to correct for the proportion of GPI-1A in the chimaeric placentas.

ANALYSIS OF CHIMAERIC CONCEPTUSES

A series of 39 conceptuses was produced (Table 4.2), which included one set of twins, a pair of conceptuses with a fused placenta and five non-chimaeric conceptuses. The five non-chimaeric conceptuses were excluded from the study (Table 4.3), as were the twins, since one set of extaembryonic membranes was shared between the two conceptuses. Although the fused placentas were separated, it was not possible to ensure that the separation was accurate so these conceptuses were also excluded from the study. The remaining 28 separate chimaeras were dissected into the component tissues. The tissues were assayed for GPI-1A activity and the results were analysed statistically. The correction factor of 45% was applied to the observed percentage of GPI-1A in the placenta to allow for the proportion which was maternally derived. In some cases, the average figure of 45% overcorrected the observed value resulting in a negative figure. The negative values were used for the statistical analysis, but were treated as zero for plotting figures. The placenta of conceptus XE-26 was lighter than average and had a high fetus/placenta ratio (joint second with 2.00 compared to a mean of 1.25 with a range of 0.67-2.50). The estimated proportion of GPI-1A was also very low (-41.5%, 22.0% uncorrected), so it seems probable that as with the conceptus in the control series, the placenta had lost the outer maternal layer.

Histograms were plotted of the distributions of the % GPI-1A for each of the tissues analysed in the chimaeric conceptuses (Fig. 4.3). The histograms show that for the fetus, amnion, yolk sac mesoderm and parietal endoderm the distributions are slightly skewed in favour of a higher percentage of GPI-1A (AF_2 cells), but the yolk sac endoderm distribution shows more equal proportions of GPI-1A and GPI-1B. Using the corrected values, the distribution of the placenta is U-shaped, with only a few

Table 4.3 % GPI-1A in tissues of $12^1/_2$ day conceptuses which were excluded from the statistical analysis.

Chimaera Ref.	% GPI-1A							
	F	A	Y.S. meso	Y.S. endo	P. endo	Placenta	Placenta corr-1*	Placenta corr-2*
Conceptuses with fused placentas								
XE-38	67	56	67	78	91	87	77	77
XE-39	50	53	44	43	83	35	-18	0
Twins within a single amnion								
XE-18a	89	84	82	31	18	44	-3	0
XE-18b	98							
Non-chimaeric conceptuses								
XE-30	0	0	0	0	0	44	-2	0
XE-1	100	100	100	100	100	100	100	100
XE-7	100	100	100	100	100	100	100	100
XE-10	100	100	100	100	100	100	100	100
XE-36	100	100	100	100	100	100	100	100

Abbreviations: F, fetus; A, amnion; Y.S. Meso, yolk sac mesoderm; Y.S. Endo, yolk sac endoderm; P.Endo, parietal endoderm.

* Placental value was corrected to allow for 45% of maternal GPI-1 in the placenta.
Placenta corr-1: %GPI-1A = [(observed %GPI-1A - 45) x 100] / (100 - 45)
Placenta corr-2: the negative values are treated as 0.

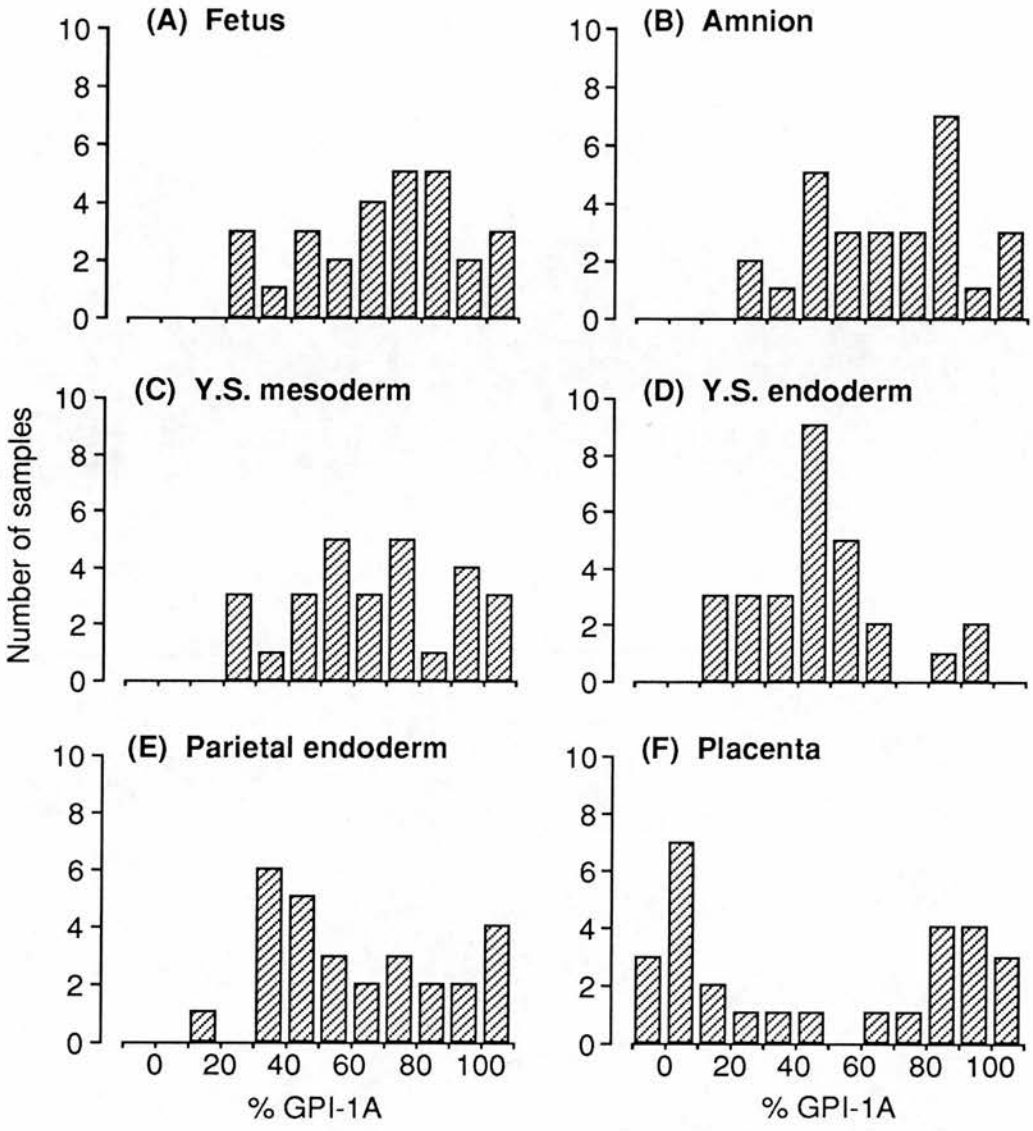


Fig.4.3 Distributions of %GPI-1A in the six tissues analysed from 28 diploid↔diploid (series XE) chimaeric conceptuses. Tissues with either 0% or 100% are shown separately at either end of the distribution.

Table 4.4: Probability (P) values from Mann-Whitney U-tests, comparing %GPI-1A in different tissues of 28 chimaeric conceptuses.

	Amnion	Yolk Sac mesoderm	Yolk Sac endoderm	Parietal endoderm	Placenta (corrected)
Fetus	0.8722	0.7304	*<0.0001*	0.2752	*0.0299*
Amnion		0.8632	*<0.0001*	0.2701	*0.0336*
Yolk Sac mesoderm			*<0.0001*	0.3066	*0.0376*
Yolk Sac endoderm				*0.0023*	0.6133
Parietal endoderm					*0.0482*

Differences in mean % GPI-1A between tissues were considered to be statistically significant when $P<0.05$ (shown in italics).

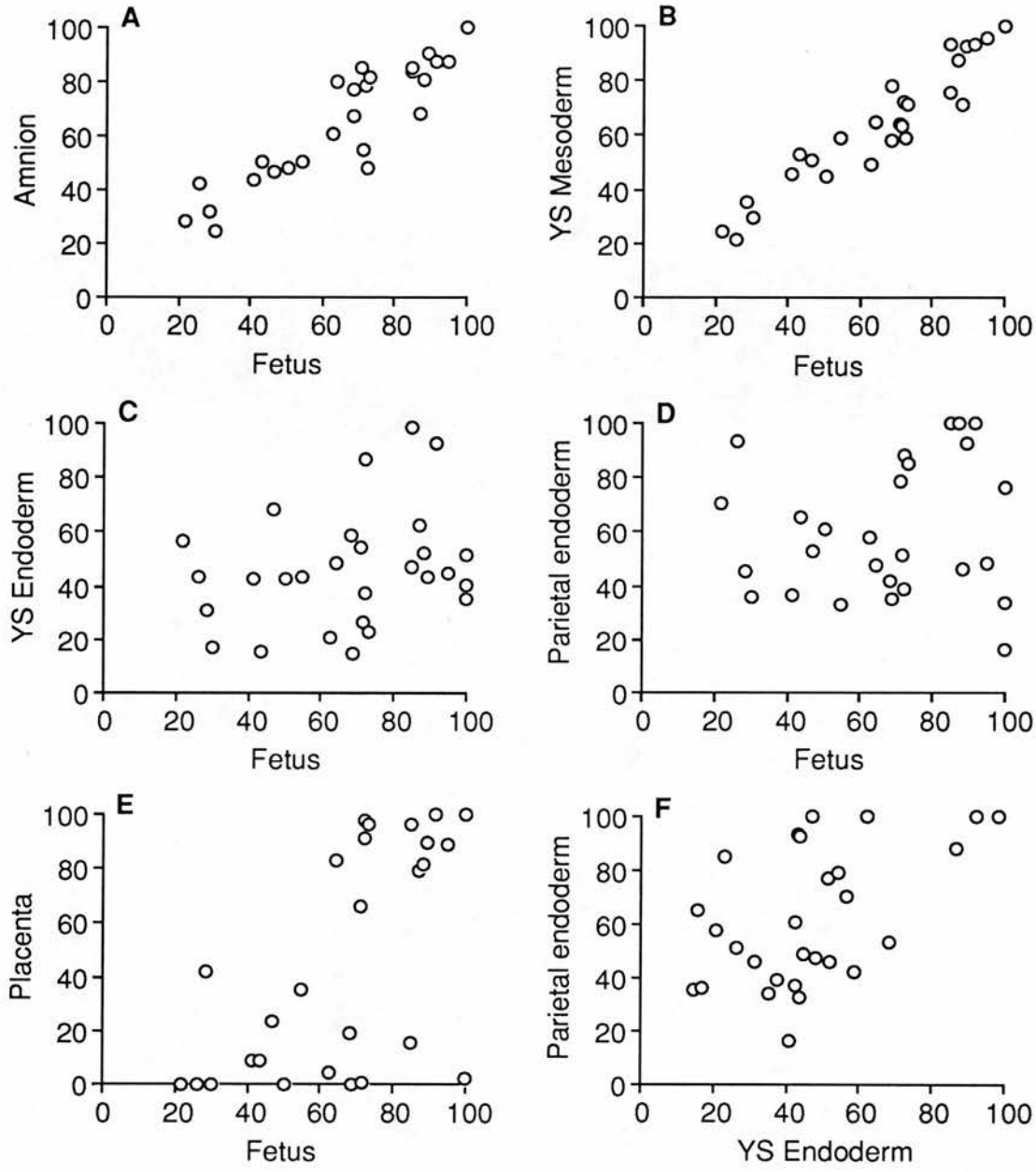


Fig.4.4 Graphs showing the relationships in %GPI-1A between pairs of tissues from diploid↔diploid (series XE) chimaeric conceptuses. The %GPI-1A in the fetus was positively correlated with that in the amnion (A), yolk sac mesoderm (B) and with the placenta (E) but not correlated to the yolk sac endoderm (C) or parietal endoderm (D). The %GPI-1A in the two primitive endoderm derivatives (yolk sac endoderm and parietal endoderm) was also positively correlated (F). See Table 4.5 for significance levels.

Table 4.5: Spearman rank correlation coefficients, r (with P values below) for % GPI-1A in different tissues of chimaeric conceptuses.

	Amnion	Yolk Sac mesoderm	Yolk Sac endoderm	Parietal endoderm	Placenta (corrected)
Fetus	0.9049 *(<0.0001)*	0.9376 *(<0.0001)*	0.2364 (0.2259)	0.1310 (0.5063)	0.6578 *(0.0001)*
Amnion		0.9219 *(<0.0001)*	0.1790 (0.3621)	0.1091 (0.5805)	0.6290 *(0.0003)*
Yolk Sac mesoderm			0.2769 (0.1538)	0.0916 (0.6428)	0.6691 *(0.0001)*
Yolk Sac endoderm				0.4882 *(0.0084)*	0.3766 *(0.0482)*
Parietal endoderm					0.2280 (0.2432)

The % GPI-1A was considered to be significantly correlated between two tissues when $P<0.05$ (shown in italics).

showing a balance of GPI-1A:GPI-1B. Consequently, the standard deviation for the corrected placentas is higher than other tissues (Table 4.2). Statistical analysis with the Kruskal-Wallis test showed that there was significant heterogeneity in the mean %GPI-1A among tissues (Kruskal-Wallis statistic=14.742; P=0.012). Further analysis with the Mann-Whitney U-test on data from pairs of tissues (Table 4.4) confirmed the visual impressions from Fig. 4.4 and showed that the mean GPI-1A was similar in the fetus, amnion, yolk sac mesoderm and parietal endoderm but differed from those in the yolk sac endoderm and the placenta. The statistical significance was higher for the difference between the yolk sac endoderm and the other four tissues than between the placenta and these other tissues. The Mann-Whitney test showed no significant difference, in the mean % GPI-1A, between the yolk sac endoderm and the placenta but Fig. 4.3 shows that, while the means are similar, the two distributions differ remarkably.

The relationships in the % GPI-1A between pairs of tissues are shown in Fig 4.4 and statistical correlations are shown in Table 4.5 The statistical analyses show that the % GPI-1A was significantly positively correlated within the primitive ectoderm derivatives (fetus, amnion and yolk sac mesoderm) and within the primitive endoderm derivatives (yolk sac endoderm and parietal endoderm) but not between the tissues derived from these two different developmental lineages. The % GPI-1A in the placenta (corrected for maternal GPI-1A) was positively correlated with the three primitive ectoderm tissues and also reached borderline significance for a positive correlation with the yolk sac endoderm but not with the parietal endoderm.

4.3 DISCUSSION

The distributions for the fetus, yolk sac mesoderm and parietal endoderm were skewed in favour of a higher percentage of GPI-1A (AF_2 cells), and when one cell population was excluded from these tissues, it was always GPI-1B. The imbalance in these

tissues may be explained by preferential allocation of the AF_2 cells to the ICM, if embryos of this strain were developmentally more advanced than their BF_2 partners (Spindle, 1982). Alternatively it may be that the AF_2 cells have a selective advantage during development. No imbalance was apparent in the yolk sac endoderm or placenta and the mean %GPI-1A in these two tissues was significantly different from the other four tissues.

This study supports conclusions previously drawn in a smaller group of chimaeras studied by West *et al* (1984). The proportions of the two cell populations were found to be highly positively correlated within the primitive ectoderm lineage (fetus, amnion and yolk sac mesoderm) and within the primitive endoderm (yolk sac endoderm and parietal endoderm), but no correlation (positive or negative) was observed between the two lineages. The agreement between this study and the previous study, although different strain combinations and enzyme markers were used, suggests that these observations have general validity. In addition they provide a useful baseline for the evaluation of chimaeras with one population of normal diploid cells, and one of tetraploid cells which may be developmentally comprised. By demonstrating how two developmentally normally (but distinct) cell populations interact in aggregation chimaeras, it is possible to determine if the presence of tetraploid cells in an embryo affects distribution of the two cell populations in a chimaera.

Two significant observations were made concerning the placenta of chimaeric conceptuses. Firstly, that the proportion of GPI-1A in the placenta correlated positively with all three of the primitive ectoderm derivatives. This was a surprising observation, because apart from the maternal contribution, the placenta comprises mainly trophoblast, which is derived from the polar trophectoderm, with only a minor contribution from the primitive ectoderm derivative (mesoderm from the allantois and chorion). There is also a small contribution of visceral and parietal endoderm (primitive endoderm). The positive correlations can be explained in two ways. There

may be an exchange of cells between the ICM and the polar trophectoderm overlying it (Handyside, 1978; Cruz and Pederson, 1985; Rossant and Croy, 1985). Alternatively, if a patch of cells of the same population straddles the boundary between the polar trophectoderm and the ICM, which may happen if extensive cell mixing does not occur (Gardner and McLaren, 1974), the positive correlation is a positional effect rather than the consequence of a shared developmental lineage.

The second significant observation of the chimaeric placentas was that the distribution of GPI-1A was U-shaped, an observation not made in other tissues. In 17/28 placentas, the minor component was 10% or less. Similar distributions have been noted by Dr.John D. West in chimaeras of a variety of strain combinations and Epstein *et al* (1984) reported a U-shaped distribution in the placentas of diploid aggregation chimaeras, where 21/26 had a minor component of 10% or less. The U-shaped distribution suggests that the main part of the placenta (trophoblast) is often derived from only one of the two aggregated embryos. This could occur if there was little cell mixing before the blastocyst stage (Gardner and McLaren, 1974), so that the polar trophectoderm was often derived from only one embryo.

The quantitative relationships established for the diploid↔diploid aggregation chimaeras provided a valuable baseline for evaluating the developmental potential of the two cell populations in chimaeric conceptuses. Since, when two embryos are aggregated to form a chimaera some degree of cell mixing occurs during development and different proportions of each cell type contributes to each cell lineage, this preliminary control experiment was also useful in confirming the previously established cell lineage relationships in aggregation chimaeras (West *et al*, 1984). These experiments also demonstrated that in the genetic combination used (AF_2↔BF_2), there was a bias towards the AF_2 population, caused by differences between the two strains of mice used. When the tetraploid↔diploid chimaeras were produced, the tetraploid embryos were created from AF_1xCMA embryos (see

appendix X), not AF_2 embryos. The knowledge that in $AF_2 \leftrightarrow BF_2$ chimaeras the bias was toward the AF_2 population suggested that in tetraploid↔diploid chimaeras, distribution effects caused by the presence of tetraploid cells would not be masked by the overall population bias.

However, when the tetraploid↔diploid chimaeras were generated, the ß-globin transgene was incorporated into the tetraploid cell population, and CF_1 females (see appendix X) were used as recipients (for details see Chapter 5.2). For these reasons, the chimaeras described in this chapter were not the perfect controls. In short, three groups of chimaeras were generated and are discussed in this thesis;

(i) 2n (no transgene)↔2n (BF_2), which are described in this chapter (series XE),

(ii) 2n (with transgene)↔2n (BF_2), which is a better control group (this group, series XL, was generated by Jean Flockhart, Margaret Keighren and John West as part of another study, and is described briefly in Chapter 5),

(iii) 4n (with transgene)↔(BF2), the experimental chimaeras described in Chapter 5.

CHAPTER 5

TETRAPLOID↔DIPLOID CHIMAERAS

5.1 INTRODUCTION

Many studies of experimentally induced tetraploidy have shown that it is possible for tetraploid mouse embryos to survive beyond the preimplantation stage (see Chapter 1.1). The developmental potential and morphology of tetraploid conceptuses observed in studies carried out by different groups have differed widely with the combination of strains used. In many instances, including the study described in Chapter 3, the tetraploid conceptuses were retarded, with a small or absent embryonic region, but with well developed ectoplacental cone and extraembryonic membranes.

Tarkowski *et al* (1977) determined cytogenetically that a proportion (approximately 20%) of the conceptuses they analysed were 4n/2n mosaics. Tetraploid cells were observed in the fetus of the mosaic conceptuses at a very low level, while in the membranes contributions of up to 50% tetraploid cells were seen. This is an interesting observation, since in tetraploid conceptuses, the extraembryonic membranes seem to be the least affected structure. Graham (1971) fused diploid blastomeres to one cell of 2-cell embryos, creating embryos with one diploid and one tetraploid cell. There was very little contribution of tetraploid cells to the resulting fetuses. Nagy *et al* (1987) demonstrated by aggregating about 15-20 diploid embryonic stem (ES) cells between two 4-cell tetraploid embryos that the tetraploid cells contributed strongly to the extraembryonic lineages but very little to the fetus. This effect may have been due to the inability of the ES cells to colonise the primitive endoderm and trophectoderm lineages well, but analysis of diploid↔ES↔diploid controls generated by Nagy *et al*

and of ES injection chimaeras by Beddington and Robertson (1989), have shown contribution of ES cells to the yolk sac endoderm and trophectoderm.

The experiments of Lu and Markert (1980) proved that it is possible to produce viable tetraploid↔diploid chimeric mice. They found abnormal fetuses at 10 days of gestation, and at birth (which did not survive). Two tetraploid↔diploid chimaeras were born alive, judged to be 50% and 10% tetraploid by pigment in the coat. Postmortem karyotype analysis performed on the 50% chimaeric animal 27 days *post partum* revealed a mere 3% of tetraploid cells in the bone marrow of the chimaera. There are several explanations for this finding. Firstly that there was a variation in the distribution of tetraploid cells in various tissues of the chimaeras (*ie.* more in the melanocytes than the bone marrow). Lu and Markert could have determined whether this was the case by systematically studying a wider range of tissues, from the chimaera, rather than only marrow from a variety of bones. Since they were using cytogenetic methods for identifying tetraploid cells, there may have been technical reasons preventing this. The low percentage of tetraploid cells may reflect that tetraploid cells are at a disadvantage and are selected against during development, or thirdly that the estimated percentage of pigment in the fur is not a true representation of the proportion of tetraploid coat melanocytes.

In the coat, pigment is secreted into the extracellular spaces by melanocytes, and a single follicle in a chimaera may contain two independently derived populations of melanocytes (McLaren, 1976). Individual hairs in a chimaera may be fully pigmented, lacking in pigment, or patchy, which may lead to an inaccurate estimation of the percentage of pigmented cells. However, overall the percentage pigmentation in the coat is a fairly good guide to the extent of chimaerism in other tissues.

Tetraploid↔diploid chimaeras were used to try and assess the developmental potential of tetraploid cells in both the fetus and extraembryonic membranes of conceptuses at

$12^1/_2$ days of gestation. It was hoped that by using the electrophoretic variant GPI, which can be assayed in both the tetraploid and diploid populations simultaneously (see Chapter 2.3), and by studying a wide range of tissues, that some of the problems encountered by Lu and Markert could be overcome, and the lineages in which tetraploid cells persist could be determined. By studying the tissues colonised by tetraploid cells, and the relationships between tissues, it was possible to suggest several mechanisms to account for the distribution of tetraploid cells in a chimaeric conceptus.

Because one of the strains the strains (CMA; see appendix XI) to be used in the generation of the tetraploid↔diploid conceptuses carried a transgene, to allow *in situ* analysis, a third control [(AF_1xCMA)↔BF_2] was created which was more similar to the tetraploid↔diploid chimaeras than the AF_2↔BF_2. Comparison of the (AF_1xCMA)↔BF_2 chimaeras with; (i) the AF_2↔BF_2 chimaeras (see Chapter 4) allowed comment on whether the presence of the transgene significantly affected the proportions of the two cell populations in the chimaera; (ii) the tetraploid↔diploid chimaeras indicated what effect the presence of tetraploid cells in the chimaera was having not only on the distribution of the two cell populations, but also on the gross morphology of the conceptuses.

Reports in the past have suggested that during early development, the inside cells of mouse embryos tend to give rise to the inner cell mass, while the trophoblast is thought to originate from the outside cells. Kelly *et al* (1978) observed that the cell division is asynchronous in cleavage stage mouse embryos, and the first cells to divide away from the 4-cell stage are disproportionately allocated to the ICM. At the 8-cell stage the blastomeres of an embryo become polarized, and depending on the orientation of the plane of cleavage with respect to the polar axis of the cell, will divide into either two polar cells (relatively large outside cells) or one polar and one apolar (relatively small inside cells) cell (Pickering *et al*, 1988). Early dividing cells divide

more frequently into one polar and one apolar cell, and therefore contribute more to the ICM than later dividing cells. This evidence seems to suggest that blastomeres dividing ahead of others may have a selective advantage to become inside or apolar cells. The frequency with which blastomeres contribute daughter cells to the inner cell mass appears to be related to the number of contacts they have with adjacent cells (Graham and Lehtonnen, 1979), and if early contact is complete, that is if a cell is completely enclosed, it becomes an apolar cell.(Pickering *et al*, 1988). To increase the number of cell contacts, it is more favourable for a small cell to be surrounded by large cells, and if the size of a blastomere is approximately halved with each cleavage, then the smaller cells formed by the earliest blastomeres to cleave may be forced into the inside of the cleaving embryo due to their size.

In tetraploid↔diploid chimaeras, where the tetraploid cells are, in theory, twice the volume of the diploid cells (Henery *et al*, 1992), there is the danger that the larger tetraploid cells will be excluded from the ICM purely because of their size. To try and create a control to determine whether or not cells would be eliminated from the ICM on the grounds of size, a series of diploid↔diploid chimaeras was generated by aggregating either 2-cell or 4-cell diploid embryos (which were greater in size, but also developmentally retarded) to 8-cell diploid embryos. To try and force the larger cells into the inside of the blastocysts, a third chimaeric combination was devised, where a 4-cell tetraploid embryo was sandwiched between two 8-cell diploid embryos.

5.2 MATERIALS AND METHODS

TETRAPLOID PRODUCTION

Tetraploid mouse embryos were produced by electrofusion using the method of Kaufman and Webb, (1990), which involves the degradation of the cell membrane, following exposure to electric field pulses (see Chapter 3.1). Two-cell embryos to be

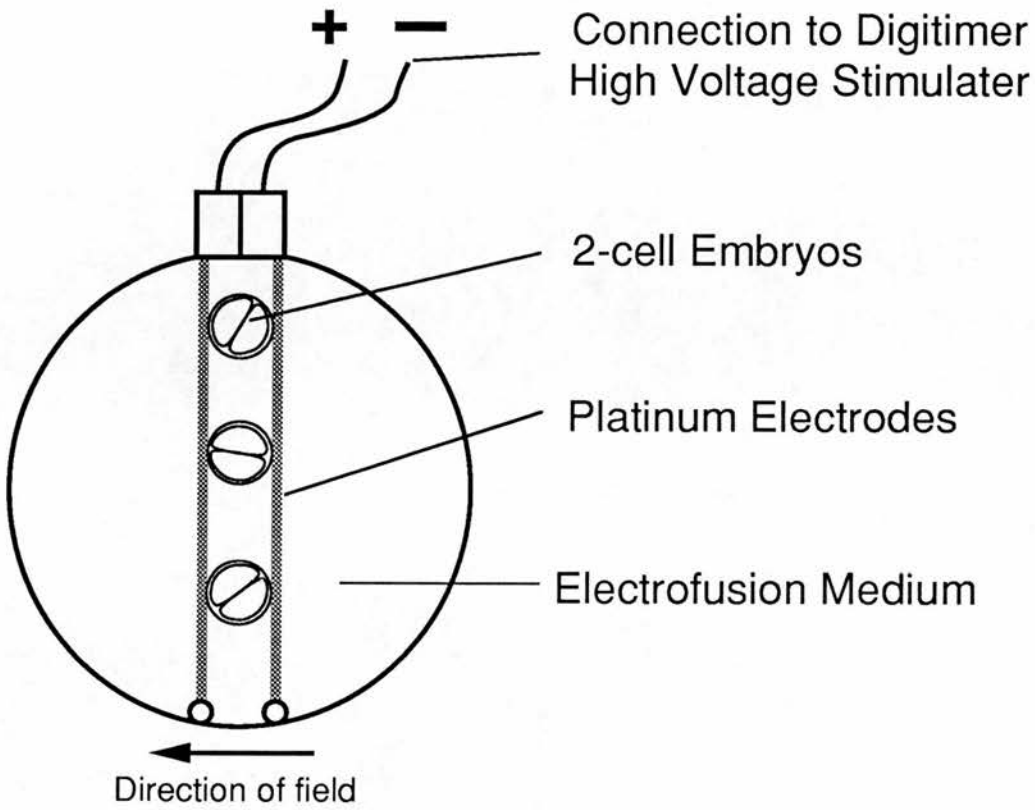


Fig.5.1 Diagram of the chamber used to perform electrofusion. The chamber was filled with electrofusion medium and 2-cell embryos were placed between the parallel platinum electrodes. Three square pulses of 50μs duration and 150v were applied across the electrodes using a Digitimer High Voltage Stimulator.

fused were collected from superovulated females at $1^1/_2$ days (see appendix II) and incubated in drops of M16 culture medium under paraffin oil at 37°C in 5% CO_2 in air.

The electrofusion apparatus consisted of a chamber with two perpendicular platinum electrodes embedded into the floor 180μm apart (Fig. 5.1), filled with non-electrolyte electrofusion medium (see appendix IX),which was connected to a Digitimer High Voltage Stimulator (see appendix IX) (Fig. 5.2*a*). Embryos at the two cell stage (Fig. 5.2*b)* were randomly orientated between the electrodes (Fig. 5.2*c*). Three square pulses of 150v and 50μs duration were applied across the electrodes, which caused disruption of the cellular membrane, allowing the two blastomeres to fuse into one cell. The embryos were then washed in M16 medium and left for up to 30min for fusion to occur. Unfused embryos were subjected up to two more rounds of electrofusion. Fig. 5.3*a* shows fused tetraploid one cell embryos alongside unfused diploid two-cell embryos. The fused one-cell embryos were cultured overnight in M16 culture medium at 37°C in 5% CO_2 before being manipulated the following day.

CHIMAERA PRODUCTION

Tetraploid↔diploid chimaeras (series XG) were generated using $(AF_1 \times CMA)F_1$ embryos for the tetraploid component and BF_2 for the diploid component (see appendix XI for an explanation of the mouse strains). AF_1 females were superovulated (see appendix II) and mated to CMA males (which are derived from the transgenic strain 83: see appendix IX). Two cell embryos were collected at $1^1/_2$ days (see appendix II), and electrofusion was performed on the $(AF1 \times CMA)F_1$ to induce tetraploidy (with two copies of the ß-globin transgene per embryonic cell; see Chapter 2). Both the fused tetraploid and the diploid (BF_2) embryos were cultured overnight. On the day following electrofusion, the tetraploid embryos were either at the 2-cell or 4-cell stage of development, whereas the diploid embryos were mainly at the 8-cell stage (Fig. 5.3*b*).

Fig 5.2 *(a)* Equipment used to produce tetraploid embryos. The electrofusion chamber rests on the stage of the dissecting microscope, and is connected to the high voltage stimulator. *(b)* Two cell embryos collected for electrofusion from the oviducts of females. *(c)* Two-cell embryos positioned between the electrodes of the chamber, immediately prior to electrofusion (bar is 100μm in *b* and 500μm in *c*).

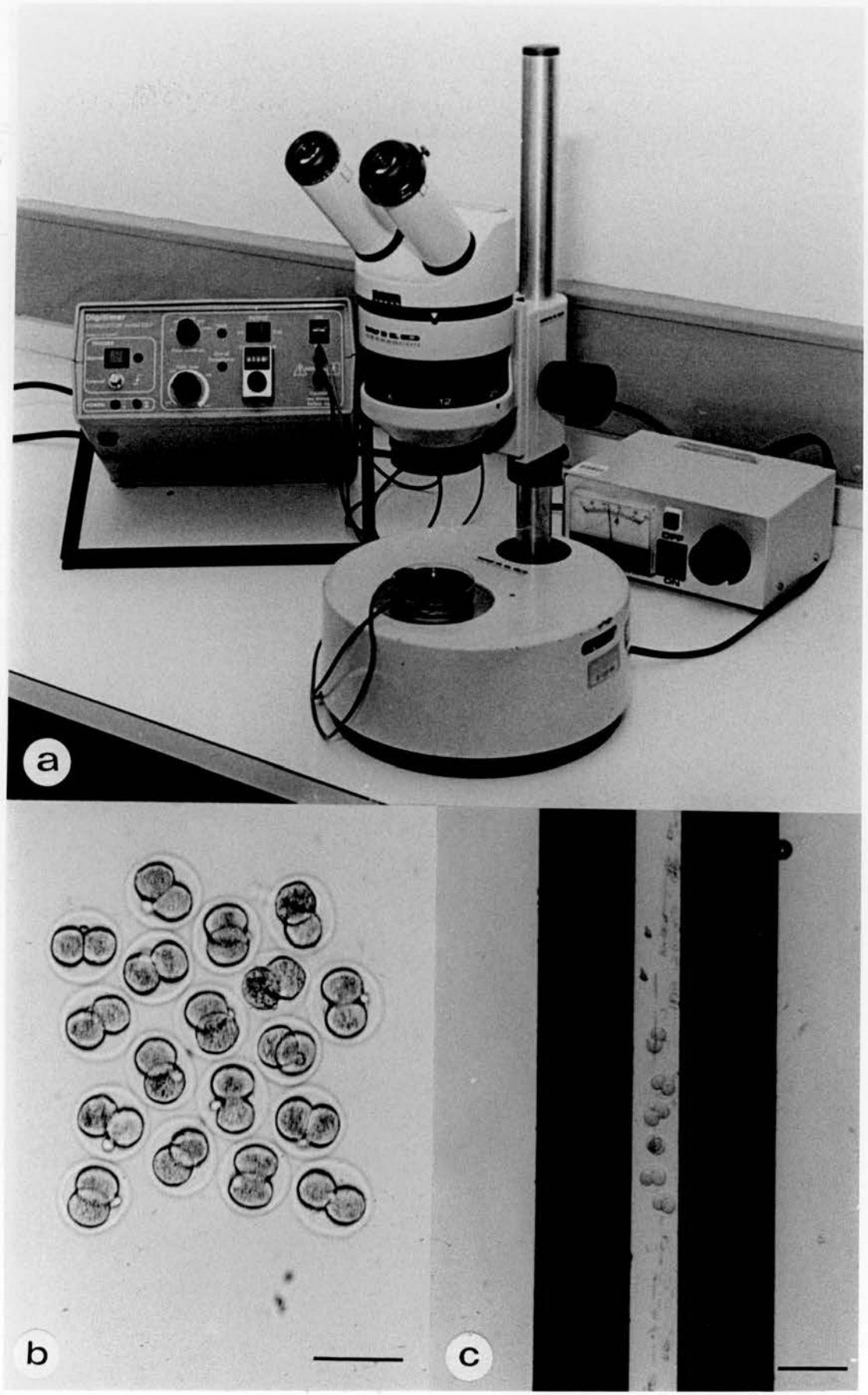

a
b
c

Fig 5.3 *(a)* Fused tetraploid one-cell embryos, along with unfused diploid embryos approximately 30 min after the electrofusion process. *(b)* Tetraploid 4-cell and diploid 8-cell embryos on the day following electrofusion, after culturing overnight. *(c)* Embryos with their zonas removed, aggregated into pairs comprising one tetraploid 4-cell embryo and one diploid 8-cell embryo. The size of the cells of the tetraploid embryos are larger than the cells of the diploid embryos. The tetraploid embryos and diploid embryos are indicated by unshaded and shaded arrows respectively. (bar is 100μm)

a
b
c

Chimaeras were produced by the same method as the diploid↔diploid control chimaeras (see Chapter 4.2 and appendix III), aggregating 4-cell tetraploid embryos to 8-cell diploid embryos and any 2-cell tetraploids to 4-cell diploid embryos (fig. 5.3*c*). After overnight culture at 37°C in M16 medium under oil at 5% CO_2, the aggregated embryos were transferred to psuedopregnant CF_1 (homozygous for *Gpi-1s*^{c}) females (see appendix IV). The embryos were mainly at the compacted morula stage rather than at the blastocyst stage: embryos subjected to fewer manipulations tended to advance to blastocyst stage at a higher frequency.

DIPLOID CONTROL CHIMAERAS

The (AF$_1$ x CMA)F$_1$↔BF$_2$ chimaeras series (series XL) were produced, dissected and analysed by Jean Flockhart, John West and Margaret Keighren as described above, omitting the electrofusion step. The 2-cell↔8-cell, 4-cell↔8-cell and 8↔4↔8-cell chimaeras (see appendices III and IV) were also produced in the same way, from the same strain combination, without electrofusion. The timing of the injections administered to superovulate the donor females was altered so that either 2-cell or 4-cell embryos from the (AF$_1$xCMA)F$_1$ strain could be collected on the same day as BF$_2$ 8-cell embryos (see appendix II). The injections to produce 2-cell embryos lagged the corresponding injections for 8-cell embryos by 24 hours and by 16 hours for 4-cell embryos. Aggregated embryos were transferred to pseudopregnant CF_1 females.

ANALYSIS BY ELECTROPHORESIS

Recipient females were sacrificed at $12^1/_2$ days of gestation, and the resulting conceptuses (tetraploid↔diploid, diploid↔diploid, 2-cell↔8-cell, 4-cell↔8-cell and 8↔4↔8-cell) were analysed in the same manner as the diploid↔diploid chimaeras (series XE) described in Chapter 4.2 The dissected tissues; placenta, trophoblast/decidual tissue, Reichert's membrane, yolk sac endoderm, yolk sac mesoderm, amnion and fetus were prepared for enzyme electrophoresis (see appendix

V). Because GPI-1C foster mothers were used, the proportion of maternal cells contributing to the trophoblast and placenta was easily determined by the presence of a third band migrating on the cellulose acetate gel plates. The maternally derived GPI-1C was not included in the data.

A rough estimate of the contribution of the BF_2 (pigmented) component in the chimaeras could be made by observing the distribution of pigmented cells in the retinal epithelium of the eye.

The homogenized tissues were subjected to gel electrophoresis as described in Chapter 2.3 (see appendix VI) to assay for the enzyme GPI. Scanning densitometry was used to determine the proportions of each alloenzyme type, GPI-1A and GPI-1B in the tissue samples. The proportion of GPI-1A:GPI-1B was used to estimate the contribution of each strain type to the resulting chimaera. For tetraploid↔diploid chimaeras, the percentage of tetraploid cells will be overestimated by the percentage of GPI-1A because each of the tetraploid cells will have approximately twice as much GPI as a diploid cell. To estimate the proportion of tetraploid cells in a given tissue it was necessary to apply a correction factor to account for this difference in cellular volume.

ANALYSIS BY *in situ* HYBRIDIZATION

The fetus head and a small piece of yolk sac were reserved from the $12^1/_2$ day tetraploid↔diploid conceptuses, fixed in 3:1 ethanol:acetic acid (see appendix VII), and processed for analysis by *in situ* hybridization to check for the presence of tetraploid cells bearing two copies of the ß-globin transgene (see Chapter 2.4 and appendix VIII).

Table 5.1: % GPI-1A in tissues of $12^1/_2$ day tetraploid↔diploid chimaeric conceptuses, ranked by %GPI-1A in the placenta.

Chimaera Ref.	% GPI-1A (tetraploid componant)						
	F	A	Y.S. meso	Y.S. endo	P. endo	Troph	Placenta
XG-12	0	0	12	21	76	93	90
XG-14	0	7	0	18	78	92	87
XG-16	0	0	0	34	54	94	82
XG-15	0	0	0	40	82	4	23
XG-19	0	0	0	68	52	0	8
XG-10	0	0	0	39	64	0	0
XG-17	0	0	0	36	20	0	0
XG-13	0	0	0	22	15	0	0
XG-9	0	0	0	15	30	0	0
XG-11	0	0	0	13	54	0	0
XG-18	0	0	0	0	17	0	0
Mean	0	0.61	1.08	27.73	49.28	25.70	26.38
S.E.M.	0	0.58	1.03	5.23	7.22	12.40	11.27
S.D.	0	1.93	3.42	17.36	23.93	41.13	37.37
N	11	11	11	11	11	11	11
Coeff Var.	N/A	316.23	316.23	62.60	48.56	160.04	141.64

Abbreviations: F, fetus; A, amnion; Y.S. Meso, yolk sac mesoderm; Y.S. Endo, yolk sac endoderm; P.Endo, parietal endoderm; Troph, trophectoderm.

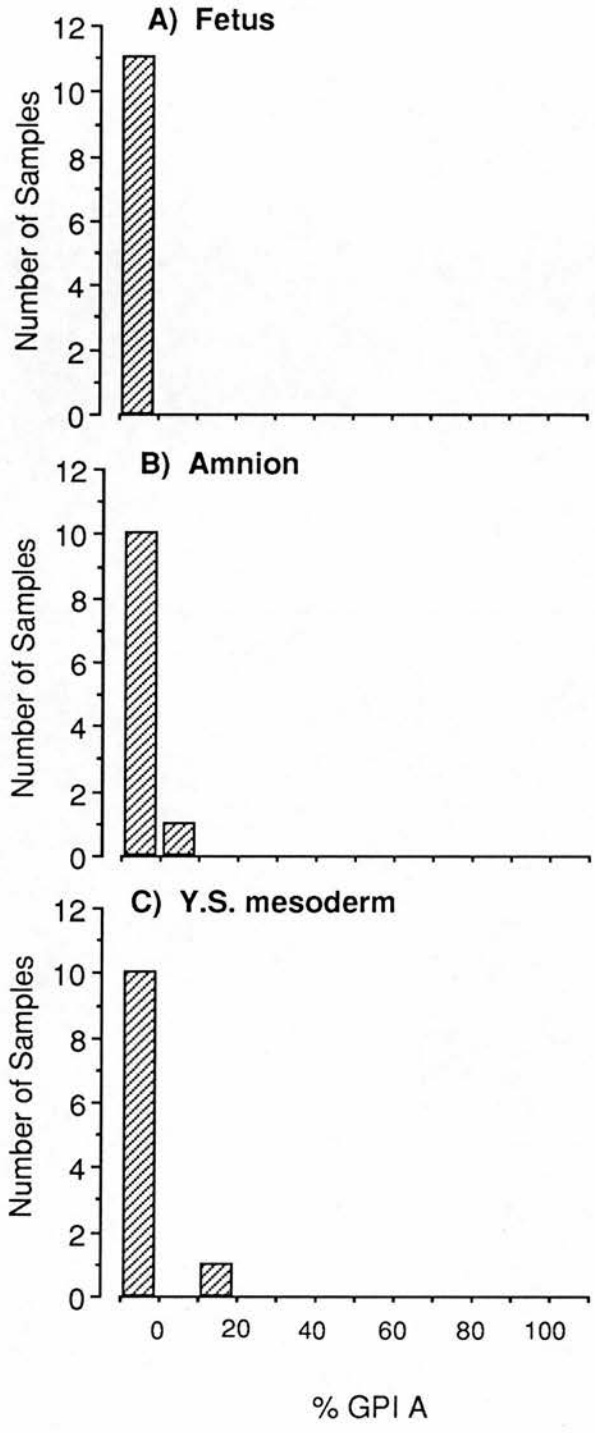


Fig.5.4 Distributions of %GPI-1A in the seven tissues analysed from 11 tetraploid↔diploid (series XG) chimaeric conceptuses. Tissues with either 0% or 100% are shown separately at either end of the distribution (continued overleaf).

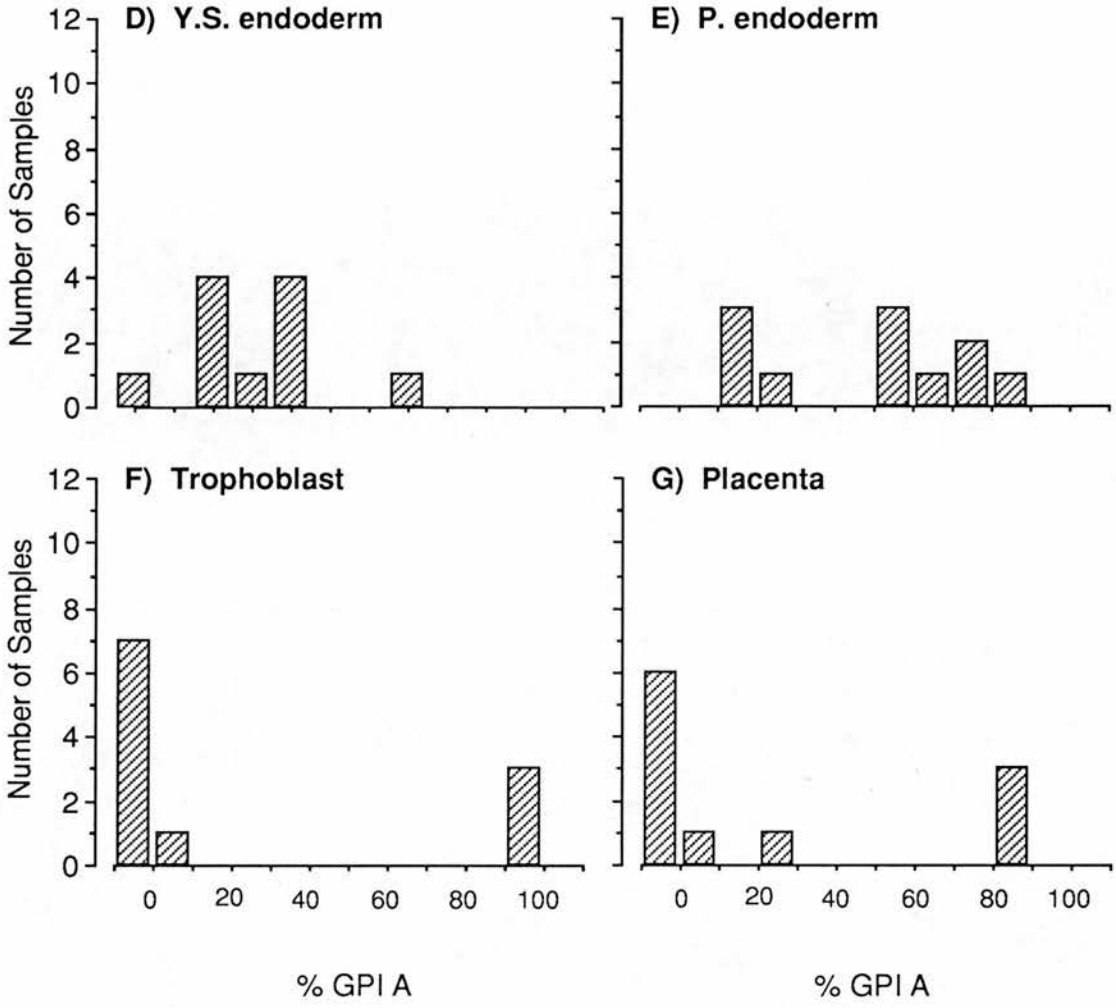


Fig.5.4 (continued)

5.3 RESULTS

TETRAPLOID↔DIPLOID CHIMAERAS

A series of 14 conceptuses was produced, 11 of which were chimaeric (Table 5.1). The distributions of the percentage GPI-1A in the chimaeric conceptuses were plotted as histograms for each of the six tissues analysed (Fig.5.4*a* & *b*). Since the $(AF_1xCMA)F_1$ embryos were homozygous for *Gpi-1s*^{a}, the presence of GPI-1A in the tissues analysed indicated the presence tetraploid cells. The histograms and the mean values in Table 5.1 show that the proportion of GPI-1A was very low in the primitive ectoderm derivatives; no contribution to the fetus, one amnion with 6.7% GPI-1A and one yolk sac mesoderm with a contribution of 11.9% GPI-1A. Both of the latter conceptuses had a high proportion of GPI-1A in the placenta, which suggest that, as in the diploid↔diploid chimaeras, there may be some degree of correlation (although not statistically significant) between the primitive ectoderm derivatives and the placenta, as discussed in Chapter 4.3. All of the tetraploid↔diploid chimaeras showed some contribution of the tetraploid cell population to the parietal endoderm, and only one conceptus (XG-18) had no contribution to the yolk sac endoderm.

The placenta displayed the U-shaped distribution of GPI-1A cells also seen in the control chimaeras, with either a large proportion of GPI-1A or no contribution at all. Although the placental distribution of GPI-1A appears to be U-shaped, it is strange in that there is either a very high proportion (around 80%) or no contribution rather than a very low contribution. Using reconstituted blastocysts, Rossant and Croy (1985) showed that 60% of the 12-14 day mouse placenta is of trophectoderm origin. The majority of the remainder is maternal in origin, with a small contribution of inner cell mass derived cells. In the diploid↔diploid chimaeras, the distribution was also U-shaped, with either a very low or very high contribution of GPI-1A cells, and it may be that if the trophoblast is either 100% or 0% GPI-1A cells, then the contribution of

Table 5.2: Estimated % tetraploid cells, after correction*, in tissues of $12^1/_2$ day tetraploid↔diploid chimaeric conceptuses, ranked by % tetraploid cells in the placenta.

Chimaera Ref.	Estimated % Tetraploid Cells						
	F	A	Y.S. meso	Y.S. endo	P. endo	Troph	Placenta
XG-12	0	0	6	12	62	87	82
XG-14	0	4	0	10	64	85	78
XG-16	0	0	0	21	37	88	70
XG-15	0	0	0	25	69	2	13
XG-19	0	0	0	51	35	0	4
XG-10	0	0	0	24	47	0	0
XG-17	0	0	0	22	11	0	0
XG-13	0	0	0	13	8	0	0
XG-9	0	0	0	8	18	0	0
XG-11	0	0	0	7	37	0	0
XG-18	0	0	0	0	9	0	0
Mean	0	0.32	0.58	17.40	36.09	23.82	22.34
S.E.M.	0	0.30	0.55	3.95	6.48	11.6	10.04
S.D.	0	1.00	1.82	13.10	21.50	38.47	33.29
N	11	11	11	11	11	11	11
Coeff Var.	N/A	316.23	316.23	75.28	59.58	161.51	149.01

* A correction factor of $(\%\text{GPI-1A}/_2)/[(\%\text{GPI-1A}/_2)+(100-\%\text{GPI-1A})]\text{x}100\%$ was applied to estimate the percentage of tetraploid cells, assumming that a tetraploid cell contains twice as much GPI-1A as a diploid cell (see text)
Abbreviations: F, fetus; A, amnion; Y.S. Meso, yolk sac mesoderm; Y.S. Endo, yolk sac endoderm; P.Endo, parietal endoderm; Troph, trophectoderm.

Table 5.3: Spearman rank correlation coefficients, r (with P values below) for % GPI-1A in different tissues of tetraploid↔diploid chimaeric conceptuses.

	Amnion*	Yolk Sac mesoderm*	Yolk Sac endoderm	Parietal endoderm	Trophoblast	Placenta
Fetus	N/A	N/A	N/A	N/A	N/A	N/A
Amnion*		-0.1000 (0.7699)	-0.2000 (0.5554)	0.4009 (0.2217)	0.3475 (0.2951)	0.4362 (0.1798)
Yolk Sac mesoderm*			-0.1000 (0.7699	0.3007 (0.3689)	0.4633 (0.1513)	0.5452 (0.0828)
Yolk Sac endoderm				0.2597 (0.4406)	0.0737 (0.8295)	0.2032 (0.5489)
Parietal endoderm					0.6490 *(0.0307)*	0.6980 *(0.0169)*
Trophoblast						0.9071 *(0.0001)*

The % GPI-1A was considered to be significantly correlated between two tissues when P<0.05 (shown in italics).
N/A: not applicable
* These tissues had only one sample each with a contribution of GPI-1A, so the correlations have little meaning.

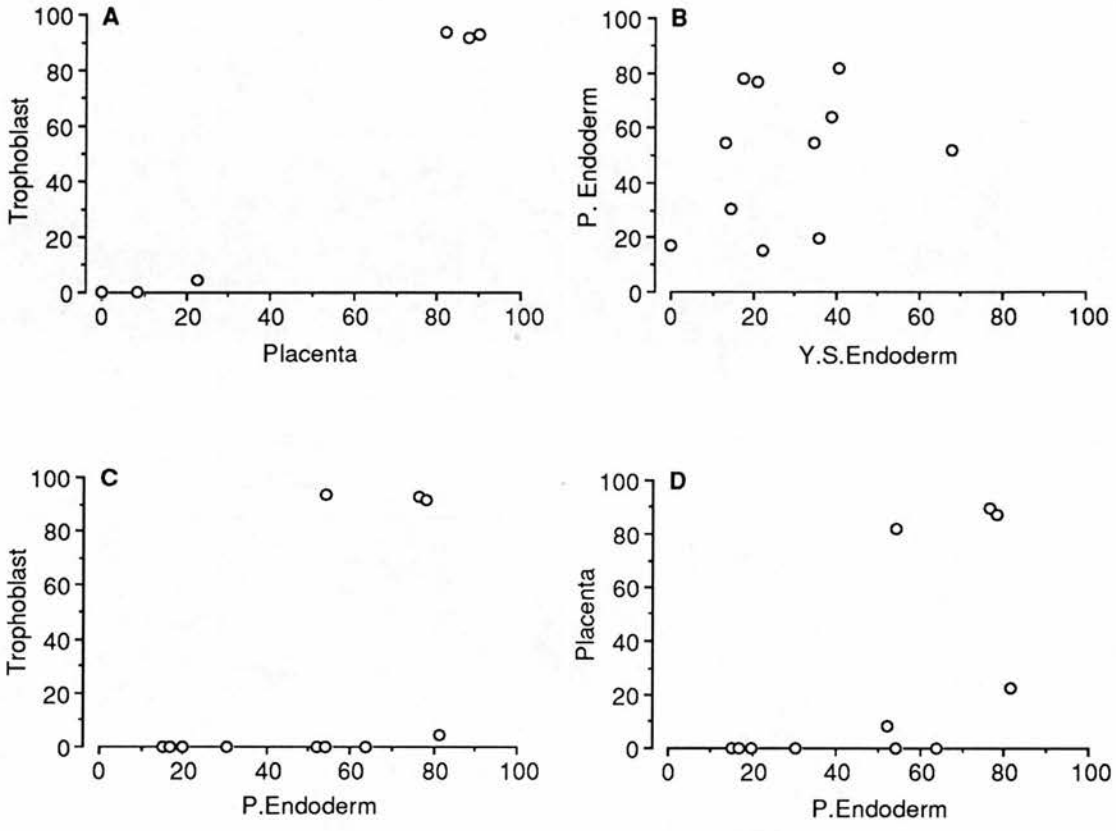
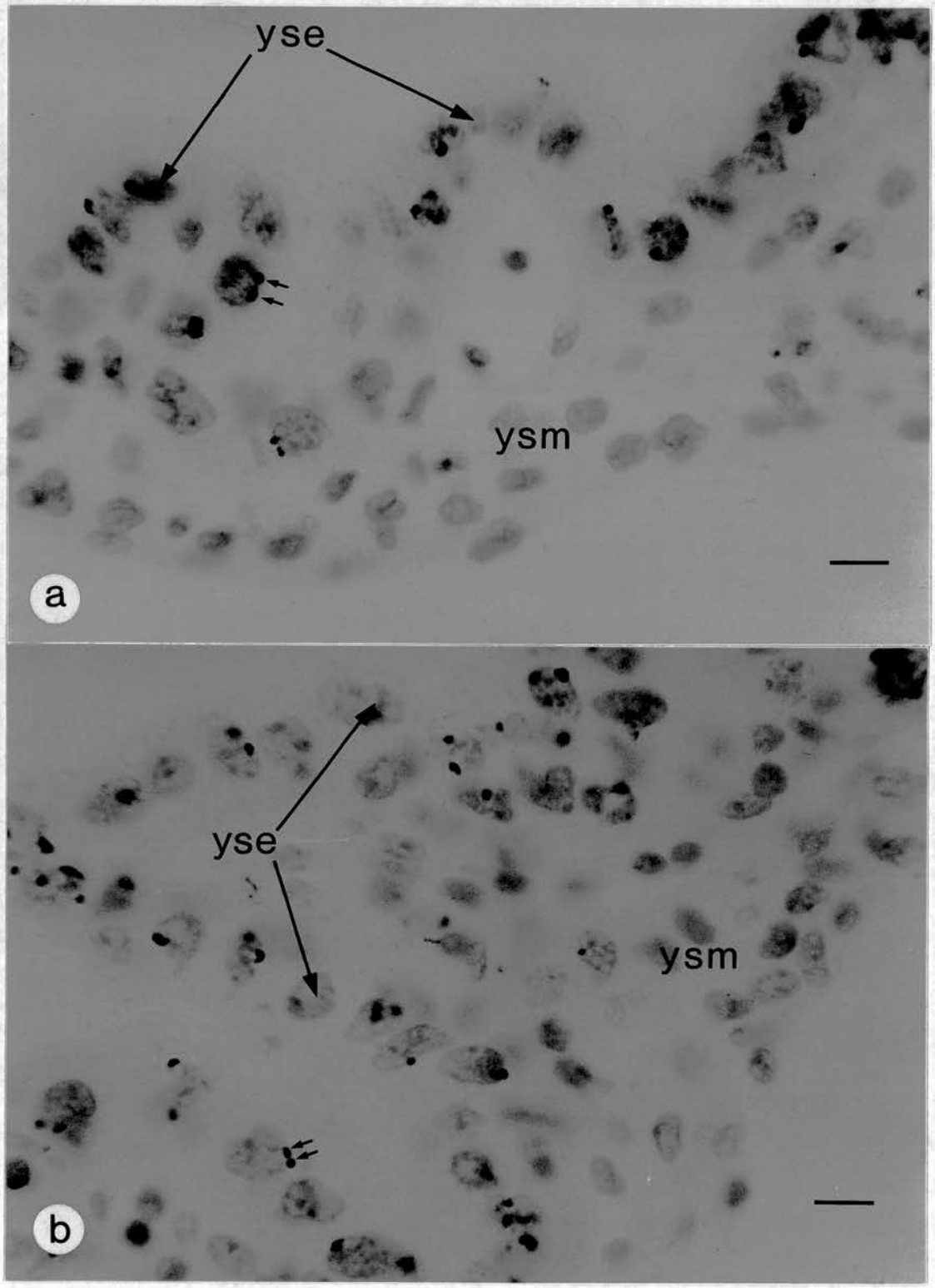


Fig.5.5 Graphs showing the relationships in %GPI-1A between pairs of tissues from tetraploid↔diploid (series XG) chimaeric conceptuses. Graphs of the primitive ectoderm derivatives were not shown since the number of samples with a contribution of GPI-1A was too low for meaningful analysis. The %GPI-1A in the two trophectoderm derivatives (trophoblast and placenta) was positively correlated (A) as in the diploid↔diploid chimaeras. The %GPI-1A in the parietal endoderm was not correlated to the other primitive endoderm derivative, the yolk sac endoderm (B), but was positively correlated to the two trophectoderm derivatives [trophoblast (C) and placenta (D)]. See Table 5.3 for significance levels.

GPI-1A cell from the ICM derivatives will alter the %GPI-1A from the placenta as a whole. However, in the tetraploid↔diploid conceptuses, there was also little or no contribution of GPI-1A in the primitive ectoderm derivatives, which means that the %GPI-1A in the placenta may have been solely from the trophoblast. The non-placental trophoblast was included in the analysis since GPI-1C foster mothers were used, and it was possible to identify and exclude the contribution of maternally derived decidual GPI from the trophectoderm derivatives.

Because tetraploid cells are twice as large as diploid cells, to estimate the proportion of tetraploid cells present in the chimaeric conceptuses rather than the percentage of GPI-1A, a correction factor was applied to the proportion of GPI-1A. The corrected values are shown in Table 5.2. The relationships in the %GPI-1A between pairs of tissues are shown in Fig. 5.5 and statistical correlations are shown in Table 5.3. The statistical analysis shows that the percentage of GPI-1A in the parietal endoderm was significantly positively correlated with both of the trophectoderm derivatives (trophoblast and placenta, which were also correlated to each other). No other tissues were statistically significantly correlated, either within or between lineages, although it was impossible to perform a meaningful statistical analysis using the primitive ectoderm derivatives, since there were no fetuses with a contribution of GPI-1A cells, and only one each of amnion and yolk sac mesoderm These correlations differ from the correlations established for the diploid↔diploid controls (series XE, see Chapter 4; series XL, see below) and from previous studies (West *et al*, 1984). There was good correlation between tissues derived from the primitive ectoderm lineage (fetus, amnion and yolk sac mesoderm) of the diploid↔diploid chimaeras. These correlations could not be established in the tetraploid↔diploid chimaeras, since there was poor contribution of tetraploid cells (GPI-1A) to the primitive ectoderm lineage. There was also a positive correlation between primitive endoderm derivatives (yolk sac endoderm and parietal endoderm) in the diploid↔diploid chimaeras, which was not seen in the

Fig 5.6 Tetraploid↔diploid chimaeric yolk sac after in situ hybridization to the ß-globin transgene. Tetraploid cells hemizygous for the transgene after electrofusion are seen with two signals per nucleus. Diploid cells show no hybridization signal. The yolk sac endoderm is a convoluted layer of cells overlying the yolk sac mesoderm. Tetraploid cells are confined mainly to the yolk sac endoderm with little contribution to the yolk sac mesoderm, which is in agreement with the data gathered from GPI analysis (bar is 10μm). Abbreviations: ysm, yolk sac mesoderm; yse, yolk sac endoderm.


yse
ysm
a
yse
ysm
b

tetraploid↔diploids. One similarity between the diploid↔diploid and the tetraploid↔diploid chimaeras, was that both groups showed a positive correlation between tissues derived from the trophectoderm lineage (trophoblast and placenta).

After *in situ* hybridization to the ß-globin transgene, no signal was seen in 7µm sections of the heads, which was expected since none of the chimaeras showed any contribution of tetraploid cells to the fetus by using the assay for GPI-1A activity. In sections of the yolk sac, the hybridization signal was confined mainly to the yolk sac endoderm, with little evidence of tetraploid cells colonising the mesoderm (Fig. 5.6*a* & *b*). This observation is also in good agreement with the GPI-1A data (Table 5.1), which demonstrated good colonisation of tetraploid cells (AF_1) to the yolk sac endoderm (mean 27.73%), but little to the mesoderm. During the course of these experiments, it was discovered that not all of the CMA males, which were used in the tetraploid embryo production were homozygous for the ß-globin transgene. Some males were found to be heterozygous and some carried no transgene. Because of this some samples had no signal after *in situ* hybridization and it was not possible to determine whether there were no tetraploid cells in the sample, or that there was no transgene present in the cells.

DIPLOID↔DIPLOID CHIMAERAS

Data from a diploid↔diploid control series of 44 (AF_1 x CMA)F_1↔BF_2 conceptuses (series XL) was provided by John West, Jean Flockhart and Margaret Keighren, which included 4 with fused placentas and 6 which were non-chimaeric. The conceptuses were assayed for GPI activity and the non-chimaeric conceptuses were excluded from the analysis (Table 5.5) as were those with fused placentas. The 34 chimaeras included in the statistical analysis are shown in Table 5.4. The data for chimaera XL-7 was incomplete and therefore was excluded from the statistical analysis. Histograms were plotted of the distributions of the %GPI-1A for each of the

Table 5.4: % GPI-1A in tissues of $12^1/_2$ diploid↔diploid day chimaeric conceptuses (series XL), ranked by %GPI-1A in the fetus (Data provided by John West, Jean Flockhart and Margaret Keighren).

Chimaera Ref.	% GPI-1A						
	F	A	Y.S. meso	Y.S. endo	P. endo	Troph	Placenta
XL-22	100	100	100	68	88	100	93
XL-40	98	94	100	70	68	90	88
XL-27	96	89	88	64	89	100	90
XL-12	95	86	89	54	71	0	6
XL-23	91	79	85	55	70	43	70
XL-2	88	85	69	59	54	17	31
XL-18	88	89	91	30	47	100	98
XL-39	73	81	65	97	100	100	100
XL-6	71	85	67	21	11	12	36
XL-21	70	71	67	51	75	74	71
XL-9	69	74	75	39	41	18	39
XL-29	68	53	57	63	80	0	6
XL-35	59	53	52	47	43	100	98
XL-31	53	39	35	11	42	0	2
XL-42	53	51	53	58	74	72	88
XL-43	49	55	46	83	68	52	15
XL-41	42	45	35	43	42	38	71
XL-13	41	41	39	40	41	38	35
XL-30	39	49	41	75	81	57	77
XL-3	39	13	15	40	9	0	12
XL-32	38	57	44	21	35	0	2
XL-7*	38	37	23	34	55		5
XL-44	36	36	34	22	42	39	25
XL-5	36	29	30	23	32	29	6
XL-34	36	43	35	48	26	14	37
XL-37	36	56	36	5	61	0	3
XL-4	33	30	24	69	63	25	42
XL-17	33	51	46	87	73	94	71
XL-14	31	42	30	73	74	95	96
XL-36	26	15	19	22	42	10	0
XL-33	25	32	33	27	29	7	40
XL-1	23	25	12	55	91	0	4
XL-24	19	29	15	18	18	77	69
XL-15	13	17	18	84	100	0	7
Mean	52.96	53.79	49.00	48.59	56.85	42.29	45.01
S.E.M.	4.42	4.24	4.48	4.08	4.25	6.73	6.22
S.D.	25.77	24.72	26.13	23.79	24.79	38.67	36.27
N	34	34	34	34	34	33	34
Coeff Var.	48.67	45.95	53.32	48.97	43.61	91.44	80.57

Abbreviations: F, fetus; A, amnion; Y.S. Meso, yolk sac mesoderm; Y.S. Endo, yolk sac endoderm; P.Endo, parietal endoderm; Troph, trophectoderm.
* The data from chimaera XL-7 was excluded from the statistical analysis since it was incomplete.

Table 5.5: % GPI-1A in tissues of $12^1/_2$ diploid↔diploid day chimaeric conceptuses (series XL) which were excluded from the statistical analysis (Data provided by John West, Jean Flockhart and Margaret Keighren).

Chimaera Ref.	% GPI-1A						
	F	A	Y.S. meso	Y.S. endo	P. endo	Troph	Placenta
Conceptuses with fused placentas							
XL-19	81	76	85	59	100	100	100
XL-20	81	79	76	40	21	85	66
XL-25	48	51	57	74	68	60	83
XL-26	26	33	27	60	18	48	35
Non-chimaeric conceptuses							
XL-8	0	0	0	0	0	0	0
XL-16	0	0	0	0	0	0	0
XL-10	100	100	100	100	100	100	100
XL-11	100	100	100	100	100	100	100
XL-28	100	100	100	100	100	100	100
XL-38	100	100	100	100	100	100	100

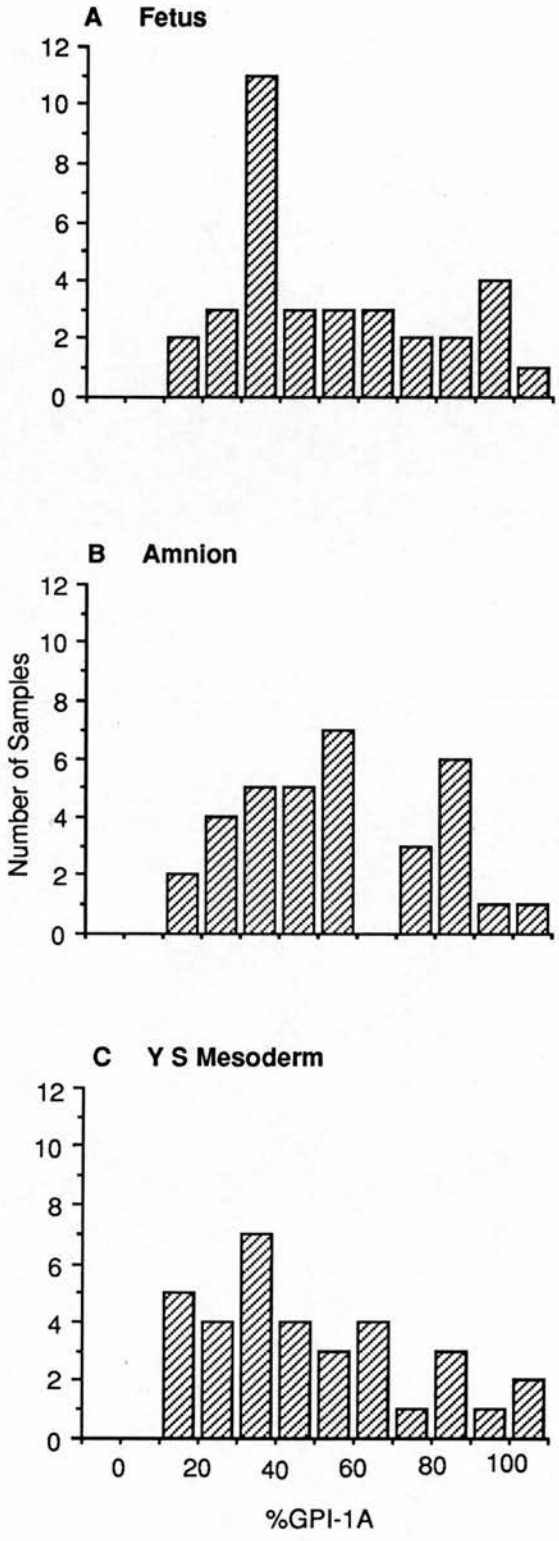


Fig.5.7 Distributions of %GPI-1A in the seven tissues analysed from 34 diploid↔diploid (series XL) chimaeric conceptuses. Tissues with either 0% or 100% are shown separately at either end of the distribution (continued overleaf).

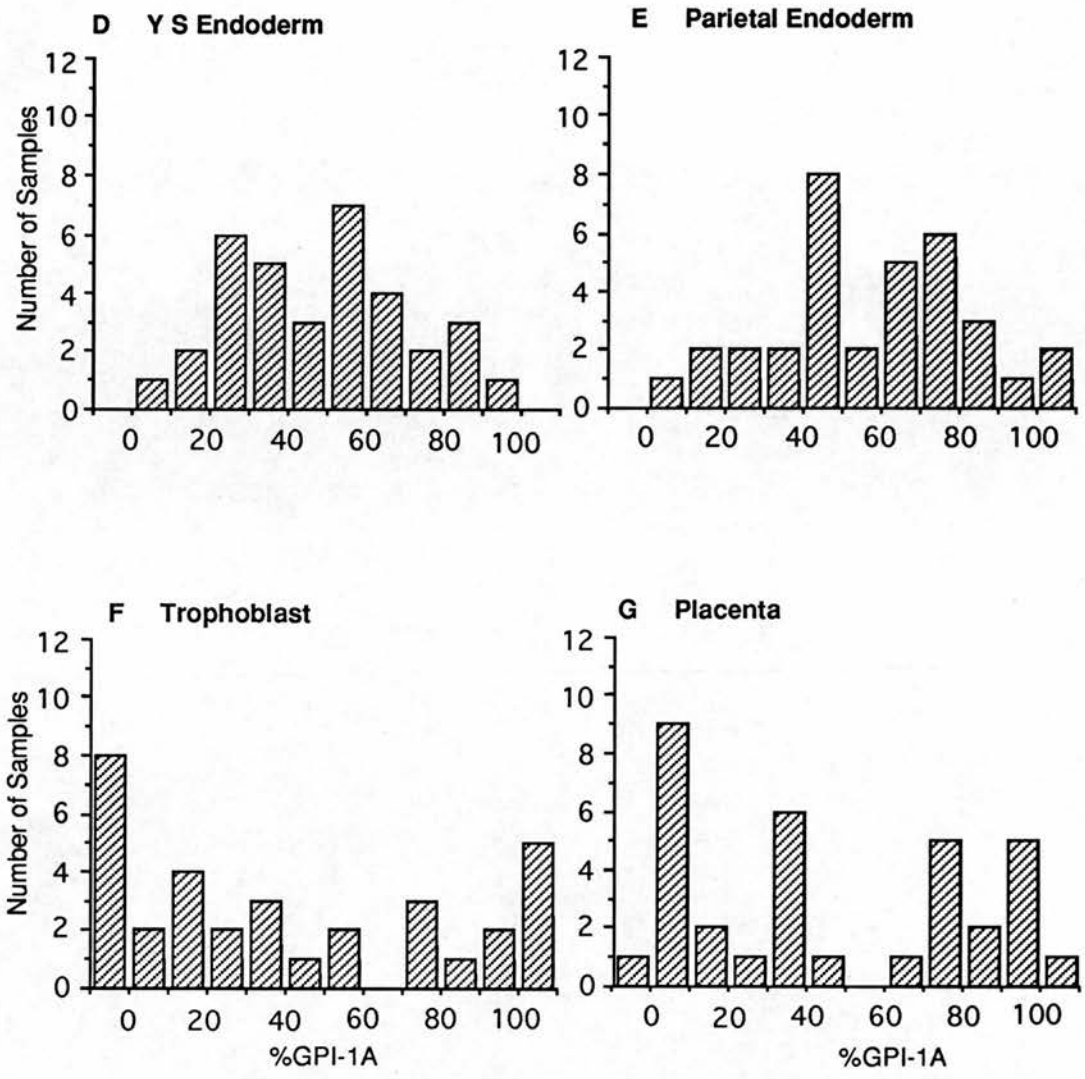


Fig.5.7 (continued)

tissues analysed in the chimaeric conceptuses (Fig. 5.7). By studying the histograms it appears that the distributions of the fetus, amnion, yolk sac mesoderm, yolk sac endoderm and parietal endoderm were balanced. The trophoblast and the placenta also appear balanced, but the distributions show the U-shape as described in Chapter 4.3 for the diploid↔diploid chimaeras (series XE). The distribution of GPI in the XE series tended more towards a skew in favour of GPI-1A. The mean %GPI-1A for each tissue (except the trophoblast as there was no XE data) was compared for the XE and XL series of chimaeras (Table 5.6) The %GPI-1A of the three derivatives of the primitive ectoderm lineage (fetus, amnion and yolk sac mesoderm) was significantly different between the series XE and XL, using Mann-Whitney U-tests. This difference may be due to skew towards GPI-1A in the primitive ectoderm lineage of the series XE chimaeras. The primitive endoderm (yolk sac endoderm and parietal endoderm) and trophectoderm derivative (placenta) were not significantly different. The %GPI-1A in different tissues of the tetraploid↔diploid chimaeras was compared to the series XL diploid↔diploid chimaeras, since the genetic background was more similar, and the XE series and XL series were significantly different in some tissues. Since the analysis was being carried out between tetraploid↔diploid and diploid↔diploid chimaeras the corrected values displayed in Table 5.2 were used so that the percentage of tetraploid cells was not being over estimated. The tetraploid↔diploid chimaeras (series XG) differed significantly in every tissue from the XL chimaeras (Table 5.6).

The statistical correlations of the % GPI-1A between pairs of tissues in the series XL chimaeras are shown in Table 5.7. The statistical analysis shows that.correlations between tissues shows the same pattern as the XE diploid↔diploid chimaeras in the there was correlation between tissues of the primitive ectoderm (fetus, amnion and yolk sac mesoderm), between tissues of the primitive endoderm (yolk sac endoderm and parietal endoderm) and between tissues of the trophectoderm (trophoblast and placenta) There was also positive correlation between the primitive ectoderm tissues

Table 5.6: Probability (P) values from Mann-Whitney U-tests, comparing mean %GPI-1A in pairs tissues from diploid↔diploid chimaeric conceptuses.

Fetus	Amnion	Yolk Sac mesoderm	Yolk Sac endoderm	Parietal endoderm	Trophoblast	Placenta*
Series XL verses XE						
0.0040	*0.0114*	*0.0007*	0.3739	0.3142	**	0.9495
Series XL verses XG (4n↔2n)						
<0.0001	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*0.0002*	*<0.0001*

Differences in mean % GPI-1A between tissues were considered to be statistically significant when P<0.05 (shown in italics).
* The placenta values for XE were corrected to account for maternal GPI-1A (see Chapter 4.3).
** No trophoblast data was available for the XE series.

Table 5.7: Spearman rank correlation coefficients, r (with P values below) for % GPI-1A in different tissues of diploid↔diploid (series XL) chimaeric conceptuses.

	Amnion	Yolk Sac mesoderm	Yolk Sac endoderm	Parietal endoderm	Trophoblast	Placenta
Fetus	0.8653 *(<0.0001)*	0.9052 *(<0.001)*	0.1808 (0.3139)	0.2228 (0.2127)	0.3554 *(0.0424)*	0.3827 *(0.0279)*
Amnion		0.9585 *(<0.0001)*	0.2199 (0.2188)	0.2915 (0.0998)	0.4184 *(0.0154)*	0.4408 *(0.0102)*
Yolk Sac mesoderm			0.2500 (0.1605)	0.2946 (0.0960)	0.4287 *(0.0128)*	0.4479 *(0.0089)*
Yolk Sac endoderm				0.7511 *(<0.0001)*	0.4053 *(0.0193)*	0.4609 *(0.0069)*
Parietal endoderm					0.2930 (0.0979)	0.2993 (0.0906)
Trophoblast						0.8945 *(<0.0001)*

The % GPI-1A was considered to be significantly correlated between two tissues when P<0.05 (shown in italics).

and the trophectoderm derivatives as also seen in the XE series. However there was a positive correlation between the yolk sac endoderm and both the trophoblast and placenta. This relationship was also seen in the series XE data between the yolk sac endoderm and the placenta, but was less significant.

Nine 2-cell↔8-cell chimaeras were generated, but none appeared by GPI analysis to be chimaeric, and in all nine cases it was the GPI-1A cell population which had been excluded, that is, the population which was derived from the 2-cell embryo. Of the 17 4-cell↔8-cell conceptuses, six were chimaeric. Table 5.8 shows that of these six, three had contribution of the GPI-1A cell population to the fetus and amnion, and four had contribution to the yolk sac mesoderm. In all of the chimaeric conceptuses there was a contribution of GPI-1A to the yolk sac endoderm and parietal endoderm (means; 20.88 and 22.92 respectively), but none had any contribution to the placenta. This is a rather surprising result, and may be an artefact of having such a small number of conceptuses or it may be another, rather extreme, example of the U-shaped distribution seen in both the diploid↔diploid and tetraploid↔diploid chimaeras. Fig. 5.8 shows a graph of the percentage of GPI-1A in the tissues of the 4-cell↔8-cell chimaeras, with the corresponding profiles of the tetraploid↔diploid and the diploid↔diploid (series XE) chimaeras superimposed. The graph shows that the diploid↔diploid chimaeras have good contribution to each of the six tissues. On the other hand, both the 4-cell↔8-cell and the tetraploid↔diploid chimaeras show a much lower proportion of GPI-1A in each of the tissues. The profile of the tetraploid↔diploid chimaeras is very similar to that obtained for the 4-cell diploid↔8-cell diploid chimaeras.

4-cell diploid embryos were sandwiched between two 8-cell embryos to try and force the (AF_1xCMA) cells into the inner cell mass of the developing blastocysts. The presence of GPI-1A activity was detected in only one of the 17 resulting chimeras, and in that chimaera the only tissue which had contribution of GPI-1A cells was the

Table 5.8: % GPI-1A in tissues of $12^1/_2$ day diploid 4-cell↔8-cell chimaeric conceptuses, ranked by %GPI-1A in the fetus.

Chimaera Ref.	% GPI-1A (4-cell componant)						
	F	A	Y.S. meso	Y.S. endo	P. endo	Troph	Placenta
C4XG-3	21	18	15	25	20	15	0
C4XG-5	12	10	8	0	21	1	0
C4XG-4	9	9	5	31	23	22	0
C4XG-1	0	0	0	14	9	0	0
C4XG-6	0	0	11	12	39	7	0
C4XG-7	0	0	0	42	25	10	0
Mean	7.05	6.28	6.60	20.88	22.92	9.20	0
S.E.M.	3.58	3.10	2.47	6.17	3.92	3.44	0
S.D.	8.78	7.59	6.05	15.12	9.59	8.42	0
N	6	6	6	6	6	6	6
Coeff Var.	124.54	120.78	91.61	72.41	41.86	91.58	N/A

Abbreviations: F, fetus; A, amnion; Y.S. Meso, yolk sac mesoderm; Y.S. Endo, yolk sac endoderm; P.Endo, parietal endoderm; Troph, trophectoderm.

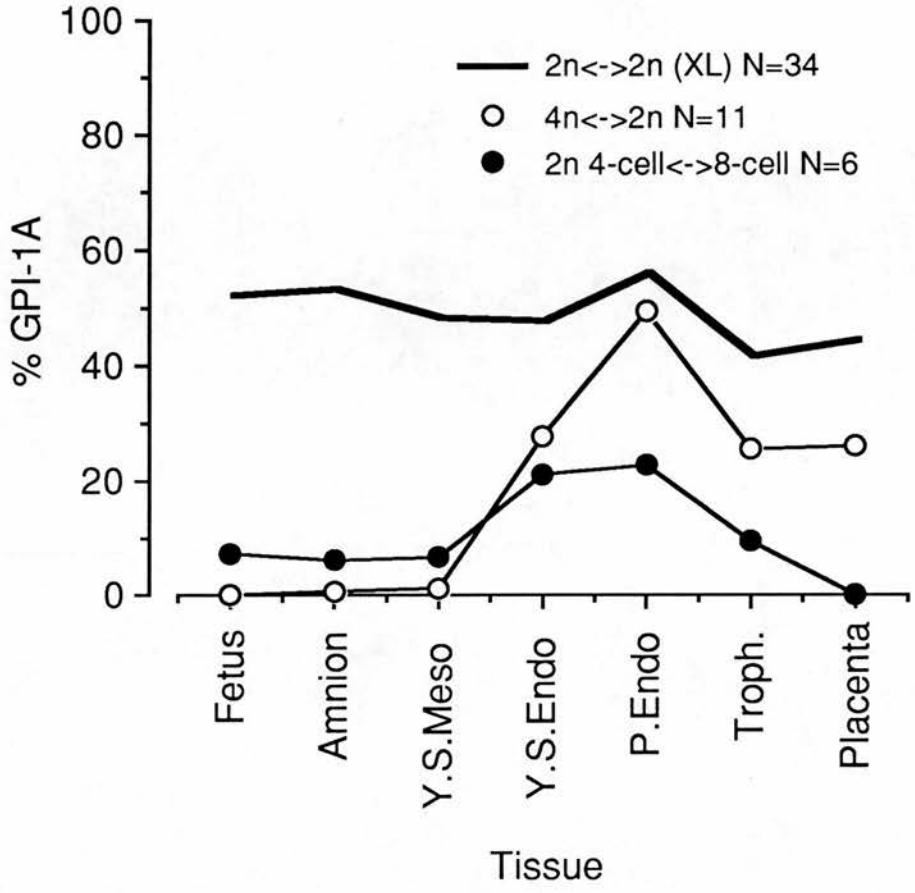


Fig.5.8 A graph showing the mean %GPI-1A in the tissues of the diploid 4-cell↔8-cell chimaeras, with the corresponding profiles of the tetraploid↔diploid and the diploid↔diploid chimaeras (series XL) superimposed. The graph shows that the diploid↔diploid chimaeras have good contribution of each cell type to each of the six tissues. The diploid 4-cell↔8-cell and the tetraploid↔diploid chimaeras show a much lower proportion of GPI-1A in each of the tissues and a marked similarity in the profile of their graphs, in that there was a very low proportion of GPI-1A (indicating cells derived from the 4-cell embryo in.diploid 4-cell↔8-cell chimaeras or tetraploid cells in the tetraploid↔diploid chimaeras) in the primitive ectoderm derivatives and a higher proportion of GPI-1A in the primitive endoderm derivatives.

parietal endoderm (24.7%). A comparable experiment was attempted previously by Lu and Markert (1980), who aggregated a tetraploid embryo between two diploid embryos. Their results were similar; there was no evidence for the presence of tetraploid cells in the resulting chimaeras. By using two diploid embryos which were genetically distinguishable, Lu and Markert were able to determine that some of their animals were diploid↔diploid chimaeras. With the strains used in this study, it was impossible to say whether the conceptuses were diploid↔diploid chimaeras or were derived from one diploid embryo.

5.4 DISCUSSION

The tetraploid cells (GPI-1A) in the tetraploid↔diploid chimaeras contributed mainly to the primitive endoderm and trophectoderm derivatives, and contributed very little to the primitive ectoderm derivatives. The relationships between the six tissues analysed do not follow the same pattern as the lineage relationships established for the diploid↔diploid chimaeras, in that; (i) there was a minimal contribution to the primitive ectoderm derivatives, (ii) the contribution to the yolk sac endoderm and parietal endoderm were not correlated. However, the trophoblast was correlated with the placenta as before. The reason for these differences may simply be that the number of tetraploid↔diploid chimaeras was too small to demonstrate statistical relationships. On the other hand, the altered relationships between tissues may reflect a genuine effect on development caused by the presence of tetraploid cells during development. There was minimal contribution to tissues of the primitive ectoderm derivatives, therefore analysis between these tissues is not meaningful.

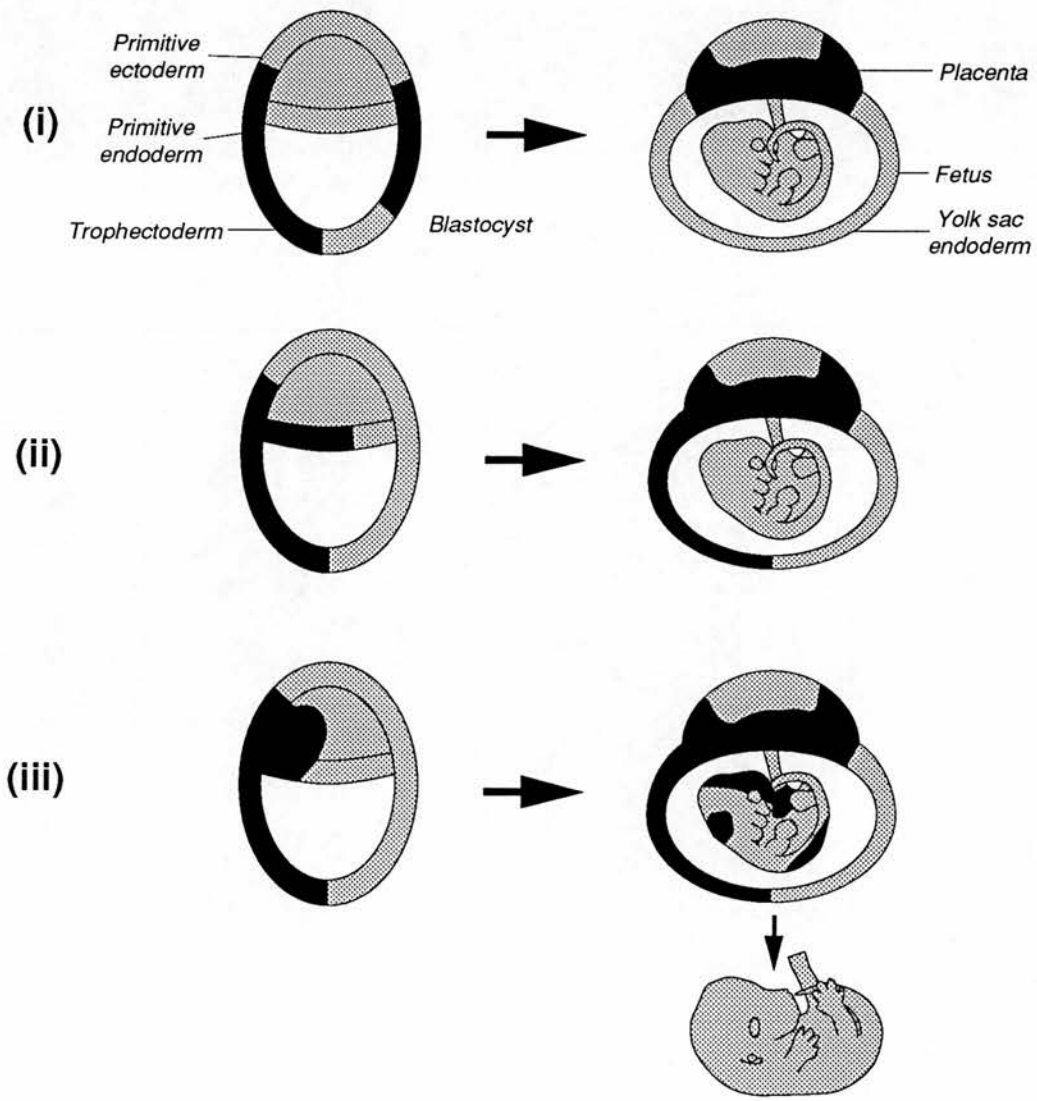
The yolk sac endoderm and parietal endoderm (primitive endoderm derivatives) failed to show the expected positive correlation. This may be significant, since all of the tetraploid↔diploid chimaeras had good contributions to the parietal endoderm. From the data analysed in Chapter 3 as well as in other studies (see Chapter 3 for

references), it appears that the parietal endoderm as well as the yolk sac and the ectoplacental cone are the least affected structures in pure tetraploids. The parietal endoderm was, however positively correlated with the trophoblast and the placenta.

It is easy to imagine that if during early development that tetraploid cells are shunted out of the inner cell mass, upwards into the polar trophectoderm, to give rise to trophoblast cell or downwards to the primitive endoderm. The lack of correlation between the yolk sac endoderm and the parietal endoderm may be due to altered cell proliferation or migration properties. The visceral endoderm must remain stuck to the ectoderm, and if the visceral endoderm cells are not adhering to it, they may become parietal endoderm. If postimplantation visceral endoderm cell are injected into blastocysts the cells usually go to the parietal endoderm (Gardner, 1974). In the 4-cell↔8-cell chimaeras the greatest proportion of GPI-1A was also in the parietal endoderm, and the 8-cell↔4-cell↔8-cell chimaeric conceptus produced had contribution of cells derived from the 4-cell embryo only in the parietal endoderm. Alternatively, the %GPI-1A in the parietal endoderm may be elevated because of contamination from the trophoblast. However, if the four conceptuses with a contribution of GPI-1A to the trophoblast are removed from the analysis, there is still no correlation between the parietal endoderm an the yolk sac endoderm (but this could simply reflect the small sample size).

The effect seen in the tetraploid↔diploid chimaeras is very similar to the distributions seen in the diploid 4-cell↔8-cell chimaeras. However, there was a greater contribution of GPI-1A cells to the primitive ectoderm derivatives of the 4-cell↔8-cell chimaeras. From these data, it seems possible that part of the reason why tetraploid cells colonise the primitive ectoderm derivatives poorly is due to the increased size of the cells. The problem may be purely mechanical and spatial in that the larger cells cannot move freely during development, or may be because of altered cell surface properties

Fig 5.9 Mechanisms to account for the distribution of tetraploid cells in $12^1/_2$ day chimaeric conceptuses; (i) abnormal cells are usually excluded from the inner cell mass; (ii) abnormal cells are usually excluded from the primitive ectoderm; (iii) abnormal cells are lost from the fetus (selection).


(i)
Primitive ectoderm
Primitive endoderm
Trophectoderm
Blastocyst
Placenta
Fetus
Yolk sac endoderm
(ii)
(iii)

destroying cell-cell interactions. The parietal endoderm cells are individual and migratory and do not form specialized intracellular junctions (Hogan *et al*, 1986), and for this reason may not be so severely affected by an increase in size.

There are three possible mechanisms to account for the distribution of tetraploid cells in the $12^1/_2$ day chimaeric conceptuses. Abnormal cells may be usually excluded from the inner cell mass (Fig. 5.9). This alternative is clearly not the case, since there was good colonisation of tetraploid cells to the primitive endoderm derivatives (yolk sac endoderm and parietal endoderm). A second, different mechanism is more likely considering the observations of this study. That is, abnormal cells were usually excluded from the primitive ectoderm derivatives. The existence of one amnion and one yolk sac mesoderm with a small proportion of tetraploid cells, and the liveborn tetraploid↔diploid mice created by Lu and Markert (1980), suggests that either this mechanism is not employed or that the process of tetraploid cell exclusion is not rigourous. Thirdly, tetraploid cells may be initially present in all three primary lineages but the abnormal cells are selected against in the fetus. The third option may seem more likely, but with this mechanism, a higher contribution of tetraploid cells in the other primitive ectoderm derivatives (amnion and yolk sac mesoderm) would be expected. The most convincing argument relying on this data would be that a combination of two of more of the above mechanisms are in play.

To aid determination of the mechanism or mechanisms acting upon the tetraploid cells in tetraploid↔diploid chimaeras, it is necessary to evaluate the distribution of tetraploid cells in chimaeric conceptuses earlier in development. A short preliminary series of tetraploid↔diploid chimaeras was produced and analysed at $7^1/_2$ days of gestation. Conceptuses were fixed and processed as described for the tetraploid conceptuses in Chapter 3.2 (see appendix VIII). Sections were cut at 7μm, to allow for the possible presence of tetraploid cells. No hybridization signal was detected in any of the sections and it was impossible to establish whether the conceptuses were

chimaeric, since GPI analysis could then not be performed. There was also the possibility, as discussed above, that males used in the experiment were not homozygous for the ß-globin transgene. Some embryos may have had tetraploid cells present, but these were not detected, as there was no target sequence in the cells to allow detection by *in situ* hybridization.

Signal throughout the conceptuses at $7^1/_2$ days of gestation would have strongly suggested that selection against tetraploid cells was occurring later in the primitive ectoderm derivatives, whereas tetraploid cells confined to the primitive endoderm and trophectoderm derivatives would have implied that an exclusion event had taken place earlier in development.

From the observations discussed it is obvious that tetraploid cells in tetraploid↔diploid chimaeras do not behave as normal diploid cells. They contribute poorly to the primitive ectoderm derivatives, and it is likely that the mechanism acting on the tetraploid cells to create the observed distributions is not a simple individual process. The data suggests that a major part of the selection against tetraploid cells is due to their increased size, but it is also feasible that altered cell-cell interaction and cell migration properties may affect the behaviour of tetraploid cells *in vivo*.

CHAPTER 6
WEIGHTS AND PHYSICAL DIMENSIONS OF CHIMAERIC EMBRYOS

6.1 INTRODUCTION

The physical dimensions of the tetraploid↔diploid chimaeras were measured and compared to those of the diploid↔diploid chimaeras. By comparing the tetraploid↔diploids to the diploid↔diploid chimaeras, it was possible to determine whether the presence of tetraploid cells in $12^1/_2$ day chimaeras affected the development of the conceptuses. By including measurement of the crown/rump length and the hind limb morphology index (McLaren and Buehr, 1990), it was possible to distinguish between developmental retardation, and reduced size due to lack of growth. The relationships between the conceptus weight, placental weight and fetal weight were included to demonstrate how the tetraploid cells affected the growth of the chimaeras. The weights and the morphology data of the diploid↔diploid conceptuses were compared to the transfer control embryos (see Chapter 4.2), to determine what effect manipulation of the embryos had on their development.

6.2 MATERIALS AND METHODS

The weight of the total conceptuses was recorded after they had been removed from the uterus of the recipient females. After Reichert's membrane, the yolk sac and the amnion were removed from the conceptuses, the weights of the placenta and the fetus were also recorded. The fetuses were placed on a squared glass grid (pitch 0.1mm; R10, Graticules) and the crown/rump length was measured using a dissecting microscope. The hind limb morphology of the fetuses was determined by the method

of McLaren and Buehr (1990). The morphology of the hind limb was used to estimate the developmental stage of the fetuses. For numerical comparison, the hind limb morphology index values were allocated a number, rather than a letter and number. For example; E6 (early stage 6)=6.0, L6 (late stage 6)=6.5, E7=7.0, L7=7.5 etc.

The measurements described above were performed on the tetraploid↔diploid chimaeras, as well as the diploid↔diploid chimaeras, (series XE, without the presence of the ß-globin transgene and and series XL, with the transgene present) and the transfer control conceptuses. The data obtained from the diploid↔diploid conceptuses was compared with that from the transfer control conceptuses (see Chapter 4.2), which were transferred directly to recipient females without manipulation.

6.3 RESULTS

DIPLOID↔DIPLOID CHIMAERAS

XE Series

Four chimaeras (XE-2, XE-5, XE-6 and XE-17) were excluded from the analysis, since data was not available for all four of the features recorded (conceptus, fetal and placental weight and crown/rump length). The remaining 24 conceptuses are shown in Table 6.1. Table 6.2 shows the relationship between pairs of data, including weights and morphological features of the chimaeric conceptuses. Statistical analysis of the data revealed a positive correlation between the conceptus weight and both the placenta weight and the crown/rump length, and that the placenta weight was also correlated with the crown/rump length. The % GPI-1A in either the fetus or the placenta was not correlated with any of the physical features of the conceptuses studied.

XL series

Data from a series of 34 conceptuses was provided by John West, Jean Flockhart and Margaret Keighren (Table 6.3) and Table 6.4 shows the relationships between pairs of features. The data shows that each of the features studied was positively correlated

Table 6.1: Weights and physical dimensions of $12^1/_2$ day diploid↔diploid chimaeric conceptuses (series XE), (ranked by %GPI-1A in the fetus).

Chimaera Ref.	Conceptus Weight (g)	Fetus Weight (g)	Placenta Weight (g)	Crown/rump Length (mm)
XE-21	0.24	0.08	0.08	8.8
XE-35	0.32	0.12	0.10	9.9
XE-16	0.36	0.12	0.10	10.0
XE-24	0.28	0.10	0.04	8.5
XE-33	0.32	0.10	0.07	9.6
XE-27	0.32	0.12	0.08	9.6
XE-19	0.20	0.04	0.06	7.7
XE-11	0.28	0.08	0.11	10.1
XE-13	0.34	0.10	0.10	9.6
XE-14	0.32	0.08	0.10	9.6
XE-8	0.20	0.10	0.06	10.2
XE-23	0.30	0.08	0.08	9.7
XE-28	0.34	0.10	0.08	9.8
XE-32	0.35	0.08	0.08	9.2
XE-12	0.34	0.10	0.10	10.0
XE-22	0.22	0.10	0.06	9.2
XE-29	0.32	0.12	0.11	9.2
XE-31	0.31	0.12	0.06	9.4
XE-26	0.28	0.12	0.06	9.3
XE-9	0.40	0.14	0.09	10.0
XE-37	0.32	0.10	0.08	9.7
XE-34	0.32	0.10	0.08	8.9
XE-15	0.34	0.08	0.10	9.9
XE-20	0.34	0.12	0.12	9.8
Mean	0.306	0.111	0.084	9.488
S.E.M.	0.010	0.014	0.004	0.118
S.D.	0.050	0.067	0.020	0.576
N	24	24	24	24
Coeff Var.	16.275	59.820	24.252	6.069

* Chimaeras XE-2, XE-5, XE-6 and XE-17 were excluded from the table since data was not available for each of the morphological features studied.

Table 6.2: Spearman rank correlation coefficients, r (with P values below) for features of gross morphology of diploid↔diploid chimaeric conceptuses (series XE).

	Conceptus Weight	Fetus Weight	Placenta Weight	Crown/rump Length
Conceptus Weight		0.3348 (0.1098)	0.5363 *(0.0069)*	0.4161 *(0.0431)*
Fetus Weight			0.1001 (0.6417)	0.2211 (0.2991)
Placenta Weight				0.4192 *(0.0415)*
% GPI-1A in fetus	-0.2296 (0.2805)	-0.1962 (0.3582)	-0.1845 (0.3880)	-0.0826 (0.7014)
% GPI-1A in placenta	-0.0145 (0.9462)	0.0075 (0.9721)	0.0065 0.9761)	0.3921 (0.581)

The morphology was considered to be significantly correlated between two features when P<0.05 (shown in italics).

* Chimaeras XE-2, XE-5, XE-6 and XE-17 were excluded from the analysis since data was not available for each of the morphological features studied.

Table 6.3: Weights and physical dimensions of $12^1/_2$ day diploid↔diploid chimaeric conceptuses (series XL), (ranked by %GPI-1A in the fetus) (Data provided by John West and Jean Flockhart).

Chimaera Ref.	Conceptus Weight	Fetus Weight	Placenta Weight	Crown/rump Length	HLM
XL-22	0.32	0.08	0.12	8.8	L6
XL-40	0.36	0.11	0.10	9.4	L7
XL-27	0.26	0.07	0.08	8.3	5
XL-12	0.27	0.11	0.09	9.6	E7
XL-23	0.41	0.16	0.08	10.8	9
XL-2	0.37	0.11	0.10	9.6	E7
XL-18	0.21	0.06	0.06	7.4	5
XL-39	0.29	0.09	0.09	9.2	E7
XL-6	0.26	0.09	0.06	8.7	E7
XL-21	0.29	0.09	0.08	8.0	L6
XL-9	0.35	0.12	0.09	9.9	L7
XL-29	0.36	0.09	0.09	9.0	E7
XL-35	0.35	0.11	0.10	9.9	L7
XL-31	0.34	0.10	0.09	9.6	L7
XL-42	0.30	0.09	0.08	9.2	E7
XL-43	0.30	0.12	0.10	9.6	E7
XL-41	0.28	0.09	0.08	9.1	E7
XL-13	0.14	0.02	0.05	5.3	4
XL-30	0.34	0.10	0.09	9.4	L7
XL-3	0.33	0.10	0.09	9.2	E7
XL-32	0.36	0.12	0.09	9.7	L7
XL-7	0.27	0.08	0.07	9.1	E7
XL-44	0.35	0.10	0.07	9.3	E7
XL-5	0.29	0.09	0.06	9.3	E7
XL-34	0.35	0.11	0.11	9.8	L7
XL-37	0.32	0.10	0.08	9.4	L7
XL-4	0.30	0.09	0.08	9.2	E7
XL-17	0.35	0.11	0.10	10.2	E8
XL-14	0.24	0.08	0.11	8.9	E7
XL-36	0.33	0.10	0.11	9.5	L7
XL-33	0.32	0.11	0.09	9.8	L7
XL-1	0.33	0.10	0.09	9.3	E7
XL-24	0.39	0.13	0.09	10.0	E8
XL-15	0.26	0.08	0.07	8.4	L6
Mean	0.311	0.097	0.086	9.2	E7*
S.E.M.	0.009	0.004	0.003	0.2	
S.D.	0.054	0.022	0.016	0.9	
N	34	34	34	34	34
Coeff Var.	17.423	23.298	18.126	10.2	

Abreviations: HLM, hind limb morphology.
* The mean could not be used for the hind limb morphology index. The mode of the data was E7, as was the median.

Table 6.4: Spearman rank correlation coefficients, r (with P values below) for features of gross morphology of diploid↔diploid chimaeric conceptuses (series XL).

	Conceptus Weight	Fetus Weight	Placenta Weight	C/R Length	Hind Limb Morph
Conceptus Weight		0.7847 *(<0.0001)*	0.5284 *(0.0013)*	0.7630 *(<0.0001)*	0.7528 *(<0.0001)*
Fetus Weight			0.5287 *(0.0013)*	0.9654 *(<0.0001)*	0.8384 *(<0.0001)*
Placenta Weight				0.5319 *(0.0012)*	0.4773 *(0.0043)*
Crown/rump Length					0.8848 *(<0.0001)*
% GPI-1A in fetus	-0.0183 (0.9180)	-0.0923 (0.6037)	0.0209 (0.9065)	-0.1657 (0.3489)	-0.2568 (0.1427)
% GPI-1A in placenta	-0.1485 (0.4019)	-0.1921 (0.2765)	0.0581 (0.7443)	-0.1796 (0.3101)	-0.1448 (0.4140)

Two features were considered to be significantly correlated between when P<0.05 (shown in italics).
Abreviations: C/R length, crown/rump length; limb morph., hind limb morphology index.

with each of the other features. The % GPI-1A in either the fetus or the placenta was not correlated to any of the physical features of the conceptuses studied.

The weights and morphological features of the XE diploid↔diploid series were compared to those of the XL series, and also to the transfer control series (Table 6.5). Student's 't' test values between features (Table 6.6), show that there was no significant difference between the mean conceptus weights, fetus weights, placenta weights or crown/rump lengths of the XE series and the XL series.

Since there were no differences between the two diploid↔diploid groups, the physical dimensions of the XE chimaeras were compared to the transfer controls to try and establish whether the embryo manipulations required to produce chimaeras affected their development. There were several significant differences between the XE chimaeras and the transfer control group. Firstly, the XE chimaeras were cultured for 24 hours, so were likely to be retarded. Secondly the XE conceptuses had twice as many cells as the transfer controls and lastly, the XE conceptuses were made up of cells from two different strains. The XE group was chosen as the comparison, since the recipient females used in both the XE series and the transfer control series were the same strain (AF_1), and neither had the ß-globin transgene present. The hind limb morphological index of the control conceptuses was slightly greater than the XL conceptuses (no data for XE) (Table 6.5). The mode of each was E7, but the median of the control conceptuses was L7 compared to E7 in the XL chimaeras. Table 6.6 shows that using student's 't' test, that the only feature which was significantly different between the control conceptuses was the crown/rump length, where the mean crown/rump length of the XE conceptuses was greater (mean ± SE for XE and control conceptuses 9.49±0.12 and 9.30±0.19 respectively). Overall, these results show that the chimaeric conceptuses were very similar in size and developmental stage to the transfer controls, so that size regulation was complete (Rands, 1986).

Table 6.5: Weights and morphological features of the diploid↔diploid chimaeras (series XE and XL) compared to those of the transfer control conceptuses.

	mean±SE C/R length (mm)	mean±SE conceptus weight (g)	mean±SE fetal weight (g)	mean ±SE placental weight (g)	HLM (mode)
2n↔2n (XE) N=24					
	9.49±0.12	0.31±0.01	0.11±0.01	0.08±0.004	no data
2n↔2n (XL) N=34					
	9.20±0.2	0.31±0.01	0.10±0.004	0.09±0.003	E7
Transfer Control					
	9.30±0.100 (N=33)	0.34±0.008 (N=33)	0.12±0.004 (N=33)	0.09±0.002 (N=34)	E7*

Abreviations: C/R length, crown/rump length; HLM, hind limb morphology index.
* median of L7.

Table 6.6: Student's unpaired 't' test values (with P values below) for the weights and morphological features of the diploid↔diploid chimaeras (series XE and XL) and the transfer control conceptuses.

	C/R length	conceptus weight	fetal weight	placental weight
XE versus XL				
	1.4257 (0.1538)	0.3593 (0.7206)	0.5878 (0.5591)	0.3472 (0.7298)
XE versus transfer control conceptuses				
	1.2168 (0.2289)	1.8351 (0.0718)	1.4030 (0.1661)	1.0940 (0.2785)

Two features were considered to be significantly different when $P<0.05$ (shown in italics).

Since the diploid↔diploid data was not significantly different, the control conceptuses were compared to the XE conceptuses (see text).
The hind limb morphology index was excluded from the Student's 't' test since the data is non-parametric.

TETRAPLOID↔DIPLOID CHIMAERAS

The physical dimensions of a series of 11 tetraploid↔diploid chimaeric conceptuses were analysed (Table 6.7). Relationships were established between the fetus, placenta and conceptus weights and the crown/rump length and hind limb morphological index, and are shown in Table 6.8. The fetus weight, placenta weight and conceptus weight were each correlated to the other two features. The hind limb morphological index was correlated to the conceptus and fetus weight, but not the placenta weight or the crown rump length. More interestingly, the percentage of GPI-1A in the placenta was positively correlated to the placenta weight, but to no other weight or morphological feature.

The weights and morphological features of the tetraploid↔diploid chimaeras were compared to diploid↔diploid conceptuses either with (series XL) or without (series XE) the presence of the ß-globin transgene. The means and standard errors of the physical dimensions of the three series of chimaeras is shown in Table 6.9, and the relationships between features is shown in Table 6.10. The only feature which was significantly different between the tetraploid↔diploid chimaeras and the diploid↔diploid chimaeras, using student's 't' test, was the placental weight. The mean placenta weight for the XG chimaeras was 0.10g ±0.006, while the XE and XL chimaeras were 0.08g±0.004 and 0.09g±0.003 respectively.

The placenta weight was the only physical feature which differed significantly between the tetraploid↔diploid and the diploid↔diploid chimaeras. The mean placenta weight was also positively correlated to the %GPI-1A in the placenta (Table 6.8), which suggested that the placentas which had more tetraploid cells were heavier. To test whether this was the case, the tetraploid↔diploid conceptuses were divided into two groups; conceptuses with a contribution of tetraploid cells to the placenta; conceptuses with no tetraploid cells present in the placenta. Table 6.11 shows the mean conceptus

Table 6.7: Weights and physical dimensions of $12^1/_2$ day tetraploid↔diploid chimaeric conceptuses (series XG), (ranked by %GPI-1A in the placenta).

Chimaera Ref.	Conceptus Weight	Fetus Weight	Placenta Weight	Crown/rump Length	HLM
XG-12	0.32	0.08	0.10	8.90	L6
XG-14	0.35	0.09	0.12	9.40	E6
XG-16	0.48	0.15	0.12	9.40	L6/E7
XG-15	0.36	0.11	0.12	9.60	L7
XG-19	0.38	0.11	0.14	10.03	5
XG-10	0.30	0.07	0.10	8.70	E7
XG-17	0.33	0.10	0.08	9.25	L7/E8
XG-13	0.25	0.07	0.08	8.40	E7
XG-9	0.25	0.06	0.08	7.50	L7
XG-11	0.28	0.07	0.07	9.30	E7
XG-18	0.35	0.11	0.10	10.01	E7
Mean	0.332	0.094	0.100	9.135	E7*
S.E.M.	0.018	0.008	0.006	0.212	
S.D.	0.061	0.025	0.020	0.702	
N	11	11	11	11	11
Coeff Var.	18.378	26.828	20.178	7.685	

Abreviations: HLM, hind limb morphology.
* The mean could not be used for the hind limb morphology index. The mode of the data was E7, as was the median.

Table 6.8: Spearman rank correlation coefficients, r (with P values below) for features of gross morphology of tetraploid↔diploid chimaeric conceptuses.

	Conceptus Weight	Fetus Weight	Placenta Weight	C/R Length	Hind Limb Morph
Conceptus Weight		0.9273 *(<0.0001)*	0.8727 *(0.0005)*	0.8428 *(0.0011)*	0.6482 *(0.0310)*
Fetus Weight			0.6545 *(0.0289)*	0.8428 *(0.0011)*	0.7275 *(0.0112)*
Placenta Weight				0.6241 *(0.0401)*	0.4337 (0.1826)
Crown/rump Length					0.5562 (0.0756)
% GPI-1A in placenta	0.5998 (0.0511)	0.3378 (0.3096)	0.6741 *(0.0229)*	0.4461 (0.1690)	0.5747 (0.0644)

Two features were considered to be significantly correlated between when P<0.05 (shown in italics).
Abreviations: C/R length, crown/rump length; limb morph., hind limb morphology index.

Table 6.9: Weights and morphological feature of the tetraploid↔diploid chimaeras compared to diploid↔diploid chimaeras with of without the presence of the ß-globin transgene.

Transgene*	mean±SE C/R length (mm)	mean±SE conceptus weight (g)	mean±SE fetal weight (g)	mean ±SE placental weight (g)	HLM (mode)
2n↔2n (XE) N=24					
_	9.49±0.12	0.31±0.01	0.11±0.01	0.08±0.004	no data
2n↔2n (XL) N=34					
+	9.20±0.2	0.31±0.01	0.10±0.004	0.09±0.003	E7
2n↔4n (XG) N=11					
+	9.14±0.21	0.33±0.02	0.09±0.008	0.10±0.006	E7

Abreviations: C/R length, crown/rump length; limb morph. index, hind limb morphology index.
* + denotes the presence of the ß-globin transgene in at least some of the chimaeras.

Table 6.10: Student's unpaired 't' test values (with P values below) for the weights and morphological features of the tetraploid↔diploid and diploid↔diploid chimaeras with or without the presence of the ß-globin transgene.

	C/R length	conceptus weight	fetal weight	placental weight
2n↔4n (XG) **versus 2n↔2n** (XE)				
	1.5376	1.3342	0.8429	2.1387
	(0.1337)	(0.1913)	(0.4054)	(*0.0400*)
2n↔4n (XG) **versus 2n↔2n** (XL)				
	0.1325	1.0924	0.3597	2.4446
	(0.8952)	(0.2807)	(0.7207)	(*0.0187*)

The hind limb morphology index was excluded from the Student's 't' test since the data is non-parametric.

Table 6.11: The mean weights and morphological feature of tetraploid↔diploid chimaeric conceptuses which had tetraploid cells present in the placenta compared to those which did not (Student's 't' test values; t with P values below).

	mean±SE C/R length (mm)	mean±SE conceptus weight (g)	mean±SE fetal weight (g)	mean±SE placental weight (g)	HLM (mode)
No 4n cells in placenta	8.86±0.35	0.29±0.02	0.08±0.01	0.08±0.005	L6
4n cells in placenta	9.47±0.18	0.38±0.03	0.11±0.01	0.12±0.01	E7
t value	1.4280 (0.1870)	2.7774 *(0.0215)*	2.0517 (0.0704)	4.2227 *(0.0022)*	

Two features were considered to be significantly correlated between when $P<0.05$ (shown in italics).
The hind limb morphology index was excluded from the Student's 't' test since the data is non-parametric.

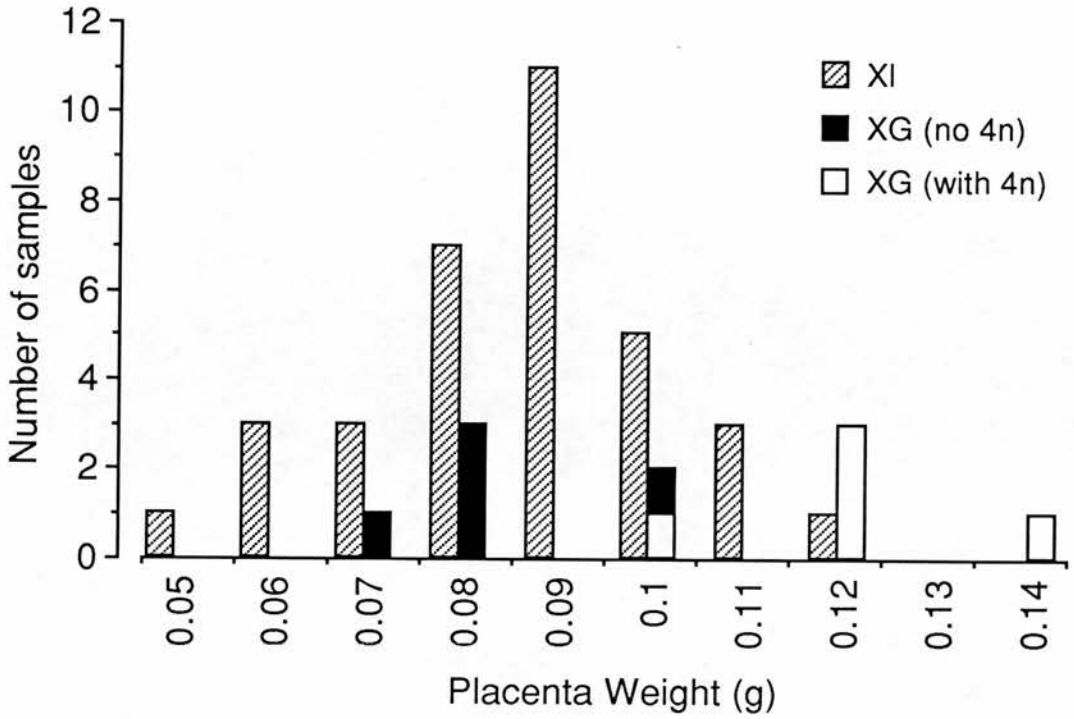


Fig 6.1 Histogram of the weights of series XG chimaera placentas with tetraploid cells compared to XG placentas with no tetraploid cells, and diploid↔diploid placentas (series XL). The distribution of placental weight was very similar in the XL series and XG series chimaeras with no tetraploid cells present, while the XG placentas with tetraploid cells were significantly heavier. See Table 6.11 for significance levels.

weight, fetus weight, placenta weight, crown/rump length and hind limb morphology for the two groups of tetraploid↔diploid conceptuses. Using student's 't' test, the placenta and conceptus weights were significantly greater in the tetraploid↔diploid conceptuses with a contribution of tetraploid cells to the placenta (Fig. 6.1).

6.4 DISCUSSION

The weight of the XE diploid↔diploid chimeric conceptuses was positively correlated to the placenta weight, although not to the fetus weight. The conceptus weight was also positively correlated to the crown/rump length. None of the other physical dimensions was correlated. The XL diploid↔diploid chimaeric conceptuses were different in that each of the features correlated with all of the other features (with the exception of the %GPI-1A in the fetus and placenta). There are two explanations for the finding that the relationships between features in the XE series of chimaeras differs from the XL series; (i) the ß-globin transgene is affecting the development of the XL conceptuses, or (ii) the measurements of one of the series were less accurate than the other. The former explanation seems less likely since there were no significant differences between any pairs of features from the XE and the XL series. The weights of the XE series of chimaeras were measured on a less sensitive and less accurate balance than the XL series, so this may be the cause of the disparity. The coefficient of variation of the weight of the XE fetuses is also much greater than that of the XL conceptuses (59.820 compared to 23.298), but lower for the crown/rump length (6.069 compared to 10.2).

The XE diploid↔diploid chimaeric conceptuses were compared to the transfer control conceptuses. The transfer controls had a greater hind limb morphology index, but a significantly lower crown/rump length. The transfer control embryos were transferred directly into the uteruses of recipient females and so were not manipulated, had intact zonae pellucidae and were not cultured overnight. The lower hind limb morphology

index of the chimaeras suggests that they are slightly developmentally retarded compared to the transfer control embryos. The lower crown/rump length of the transfer controls may be a consequence of a greater number of conceptuses per pregnancy, since a very small number of conceptuses will tend to each grow larger (McLaren and Michie, 1956), and that the chimaeras start with twice as many cells as a normal embryo.

Most of the physical dimensions of the tetraploid↔diploid chimaeras were positively correlated with each other, following the pattern of the XL chimaeric conceptuses. The features which were not correlated were the hind limb morphology index with the placenta weight and the crown/rump length. Since the hind limb morphology is a measure of the developmental stage of the conceptuses, the lack of correlation between the hind limb morphology and the placenta weight and crown/rump length may indicate that the development and the growth of the conceptus are affected differently by the presence of tetraploid cells. For instance the development may be unaffected while the size of the conceptus is altered by the tetraploid cells in some way.

The only feature of the tetraploid↔diploid conceptuses which was significantly different to the diploid↔diploid conceptuses was the placenta weight. This was an interesting discovery since the placenta weight of the tetraploid↔diploid conceptuses was positively correlated to the % GPI-1A, and so the percentage of tetraploid cells in the tetraploid↔diploid chimaeric placenta. Considering the discoveries made about the nature of the placenta in tetraploid↔diploid chimaeras, the effect of the presence of tetraploid cells in placenta was investigated. When there were tetraploid cells present in the placenta, the conceptus weight and placenta weight were both significantly greater than in conceptuses with no tetraploid cells in the placenta (Table 6.11; Fig. 6.1). There are two possible explanations for this finding. Firstly that the tetraploid cells are larger, and that they are producing more factors to aid the growth of the placenta, or

alternatively that the increase in weight is due to an accumulation of fluid in damaged tissues which have been mechanically disrupted by larger tetraploid cells. These two alternatives could be resolved by histology of the placenta. The increase in conceptus weight is probably entirely due to the increased placenta weight (McLaren and Michie, 1956).

From this data it has been shown that the overall development of tetraploid↔diploid conceptuses was not significantly different from that of diploid↔diploid chimaeras. It is therefore reasonable to suggest that the tetraploid cells in the extraembryonic tissues of the chimaeras do not affect development of the conceptuses. One tissues which did seem to be significantly affected by the presence of tetraploid cells was the placenta, where the presence of tetraploid cells resulted in a greater mean placental and consequently, conceptus weight. It is not unusual that the crown/rump length, hind limb morphology and fetus weight were not significantly different to the diploid↔diploid conceptuses, since there were no conceptuses which had a contribution of tetraploid cells to the fetus.

CHAPTER 7

DISCUSSION

7.1 THE LETHALITY OF TETRAPLOIDY IN THE MOUSE

All of the attempts to produce tetraploid mouse embryos experimentally, whichever method used, have resulted in variations in the stage of development of the tetraploid conceptuses (for references see Chapter 1.1). The resulting conceptuses are usually developmentally retarded when compared to diploid conceptuses of the same gestational age, are generally not compatible with survival to term and are grossly abnormal, although the morphology and extent of development are highly strain dependant, as discussed in Chapter 1.1.

The question which arises from this study and previous studies is, why do most tetraploid conceptuses fail to survive and develop to term? Studies in the past have focused on different stages of development, according to the viability of the strain combinations which were used. It appears that tetraploid conceptus may; (i) fail early in gestation, at the blastocyst stage, which may be a consequence of reduced cell numbers, (ii) develop to the egg cylinder stage, but display early arrested development, perhaps due to a problem with mesoderm formation, (iii) may develop to the late fetal stage, showing evidence of haemorrhages, again due to cell size, and craniofacial abnormalities, because of failure of cell migration or cell-cell interactions.

Humans tetraploid conceptuses are often discovered as empty gestational sacs, which are similar to mouse tetraploid conceptuses failing at the egg cylinder stage. Human fetuses which survive to term tend to suffer from abnormal facial features and mental

retardation. Probably a proportion of human tetraploid conceptuses also fail at the blastocyst stage but remain undetected.

Trying to explain the failure of tetraploid conceptuses at the blastocyst stage, Snow (1975) suggested that the developmental problems encountered by tetraploid embryos were more likely to be physiological and numerological than genetic. His studies of tetraploid blastocysts (Snow, 1976) showed that many of the embryos lacked a functional inner cell mass (ICM), and although the tetraploid cells were twice the volume of diploid cells, there was a reduction in the number of cells in the tetraploid embryos. It is known that a reduction in the total number of cells in a blastocyst will always affect the size of the ICM first (Tarkowski and Wróblewska, 1967). The reduced number of cells in tetraploid conceptuses may have a crucial bearing on their development. It is established that during cleavage those cells which lose contact with the external environment, and become totally enclosed by other cells are determined to form ICM (Snow, 1976). As experimentally induced tetraploid embryos have half the number of cells of diploid embryos at the same developmental age, perhaps fewer cells in tetraploid conceptuses become totally enclosed and determined as ICM cells. Eglitas and Wiley (1981) reported that tetraploidy in mouse embryos had no significant effect on the timing of preimplantation development, neither in the timing of blastocoel development nor the timing of expression of a stage dependant cell surface antigen. These findings suggests that the developmental timing of tetraploid embryos may be normal compared to diploid embryos, although the cell numbers are reduced, and that the lack of ICM may be due to cavitation occurring without enough cells for the determination event to be successful.

The tetraploid conceptuses described in Chapter 3 resembled most closely those produced by Tarkowski *et al* (1977), in that development was arrested at the egg cylinder stage. The ectoplacental cone, Reichert's membrane and the yolk sac were the

least affected structures both in the embryos described in Chapter 3 and those of Tarkowski *et al* . These observations suggest that the trophectoderm and primitive endoderm are the lineages least affected by the presence of tetraploid cells. Often there was evidence of the amnion and the allantois, which suggests that mesoderm is being produced. The embryonic ectoderm, however, usually amounted to no more than a disorganised clump of cells attached to the yolk sac, or perhaps, rudimentary hind region of the embryo. What events during development may account for this pattern? The following are purely speculative ideas to try and explain what may happen to tetraploid conceptuses during development at the egg cylinder stage.

A tetraploid conceptus has larger cells, but has fewer in number than a diploid embryo. At gastrulation, the primitive ectoderm ingresses through the primitive streak producing mesoderm. A proportion of the mesoderm emerges proximally, to become extraembryonic mesoderm (see Chapter 1.2). If the cells of the embryonic ectoderm are reduced in number, it may be that initially, the majority of the mesoderm produced accumulates in the extraembryonic region of the embryo, and goes on to form the mesoderm of the visceral yolk sac, the mesoderm of the chorion and the amnion. As the primitive streak extends, there are fewer cells to ingress, and the embryonic mesoderm is reduced. Also, perhaps the number of cells which pass through the primitive streak is genetically determined, and too many embryonic ectoderm cells ingress, leaving little ectoderm in the embryonic region. However, this theory is disproved by the presence of, an often disproportionately large, allantois, which begins to form when the mesoderm has formed a complete layer in the embryonic region (Beddington, 1992). If the suggested hypothesis were true, and the available mesoderm was recruited predominantly into forming early structures, the allantois would never be formed without evidence of embryonic mesoderm. Perhaps the cells which are seen in the embryonic region of tetraploid embryos are mainly mesoderm cells, and that is why the fetus does not develop. This is another unlikely scenario,

since Tarkowski *et al* (1977) found that the tissue in the embryonic region was competent to neural induction and most probably ectoderm.

Snow (1975) was able to produce tetraploid conceptuses which survived to the late fetal stage (one survived to term). He observed many haemorrhages in the fetuses he recovered, and concluded that they were the result mechanical stress. For instance, blood cells derived from the yolk sac forced through vessels of normal or smaller than normal size would disrupt the vessels. Another problem of cells of increased volume is that their surface:volume ratio is altered. Assuming that the cell is spherical, a two-fold increase in volume is accompanied by an increase in diameter of 26% and an increase in surface area of only 59%. The relative reduction in surface area may be limiting to the passage of metabolites and waste products out of the cell, and so may be detrimental to the cell. To restore the surface:volume ratio, a drastic change in cell shape is required, which may in turn cause morphological deformities. Changes in the surface:volume ratio, and in the cell shape, may affect the distribution of receptors and cell surface antigens, so that cell-cell interaction are disrupted or destroyed. This is an important factor when considering organs which require induction to develop normally.

The abnormalities in late fetal stage tetraploid conceptuses observed by Snow (1975) also included abnormalities of the anterior end of the neural tube and the brain. These findings were mirrored in the study of Kaufman and Webb (1990), where tetraploid embryos displayed abnormalities of the cephalic region, especially the forebrain, and extensive craniofacial abnormalities involving the forebrain and eyes. These abnormalities may be the result of failure of the migration of prechordal mesoderm into the area anterior to the notochord (Kaufman, 1991b). This failure to migrate could be the result of altered cell surface properties, or a reduction in the number of cells contributing to the mesoderm. Migration of prechordal mesoderm is thought to be

essential for normal development of the mid-facial region, and has an inductive role in morphogenesis of the forebrain. Alternatively the craniofacial feature may be caused by interference in the neural crest cell migration to the region.

It is interesting to think about the morphology of the tetraploid conceptuses arrested at the egg cylinder stage, whilst bearing in mind the fate of tetraploid cells in the tetraploid↔diploid chimaeras. There was little or no contribution of tetraploid cells to the primitive ectoderm derivatives in the chimaeras, while in the pure tetraploids, the embryonic ectoderm was reduced or absent, but the amnion, yolk sac mesoderm and allantois were generally formed (Tarkowski *et al*, 1977; Chapter 3). The yolk sac and parietal endoderm were often present in the tetraploid conceptuses, and in turn had a good contribution of tetraploid cells in the chimaeras. The same was true of the trophectoderm derivatives; trophoblast and placenta.

In conclusion, in pure tetraploid conceptuses, it appears that the derivatives of certain lineages can tolerate tetraploidy better than others. The trophectoderm and primitive endoderm lineages seem least affected by tetraploidy, while the fetus is more likely to be affected. The data seem to suggest that the developmental problems of tetraploid conceptuses may be the consequence of a lack of mesoderm, although the amnion and allantois are often complete. Perhaps the fetus is more affected by the abnormal cells because control of development is more rigid, and the tetraploid cells are not tolerated. More complex processes take place during the development of the fetus, compared to membranes, which are relatively simple structures, and it may be that tetraploidy disrupts these complex processes to a much greater extent than is possible in a simpler structure. Therefore, the membranes may appear less affected by tetraploidy.

7.2 THE CLINICAL SIGNIFICANCE OF TETRAPLOIDY

The vast majority of human tetraploid conceptuses spontaneously abort early in gestation, and may range from the typical histology of triploid partial moles to empty gestational sacs. These conceptuses are fairly similar to the mouse tetraploid conceptuses which fail to develop beyond the egg cylinder stage.

Human tetraploid conceptuses that survive to term have a wide range of abnormalities (see Chapter 1.1), which include mental retardation, limb defects and genital abnormalities. There are also commonly abnormalities of the head, face and brain, which is interesting, bearing in mind the craniofacial abnormalities reported in mouse tetraploid fetuses by Kaufman and Webb, (1990). It may be possible that the same mechanisms are acting in both the mouse and human fetuses to produce similar morphology. Alternatively, entirely different processes may bring about the same end result. More research in this direction in the mouse system may shed light on the abnormalities which occur in human tetraploid infants.

The majority of liveborn human tetraploid cases are mosaic, with a tetraploid and a normal cell line. Since many of these individuals have a high proportion of tetraploid cells in their tissues, the mouse tetraploid data, in this instance does not match with the human data. In mouse tetraploid↔diploid chimaeras there was no contribution of tetraploid cells to the fetus, so there is no direct comparison with the mosaic human cases. Perhaps it is the case that in surviving tetraploid/diploid mosaic humans, the tetraploid cells arise in the primitive ectoderm after the establishment of the three primary lineages and so can not be shunted to the outside parts (primitive endoderm or trophectoderm) of the blastocyst. This would assume that there was no great selection against tetraploid cells in the fetus. There is evidence, however, that selection against tetraploid cells, after birth may occur. Quiroz *et al* (1985) reported on a malformed

male infant who at 2 months of age was shown to have 68% tetraploidy in cultures of peripheral blood lymphocytes. Karyotype analysis carried out 6 months later revealed only 12% tetraploid cells, indicating some cell selection has taken place. This may not be unusual in blood, which originates from a stem cell population, and will be rapidly turning over.

However, the tetraploid↔diploid chimaeras may provide a much better model for another aspect of human tetraploidy. That aspect is chromosomal mosaicism, confined to the placenta, discovered during prenatal diagnosis. At the moment there are two well documented methods of sampling tissue for prenatal diagnosis. The older, more tried and tested technique is that of amniocentesis, performed in the second trimester. This method is reliable in that as little as 0.5-1% of the amniotic fluid cells cultured will be maternal in origin (Gardner and Sutherland, 1989) and that the chance of missing a fetal abnormality is around one in 4000-8000, taking into account that approximately one in 40 amniotic fluids are abnormal. The other technique is chorionic villus sampling (CVS), which is carried out earlier in gestation (first trimester), 8-10 weeks sooner than amniocentesis. For parents this is more of an advantage since it means less time to worry that the result will be abnormal, and if a termination is required, the operation is simpler earlier in gestation.

One disadvantage of CVS, however, is that there are many discrepancies between the karyotypes gained from chorionic villi and those obtained from amniotic fluid cells sampled later in pregnancy and from fetal tissues (Kennerknecht *et al*, 1991). Most of these discrepancies concern confined placental mosaicism, mainly involving trisomies, but structural rearrangements, monosomies and polyploidy have also been reported (Kalousek *et al*, 1991). The Medical Research Council European Trial of chorion villus sampling (1991) reported that there was a greater proportion of abnormal diagnosis in CV samples (5.2%) compared to amniocentesis (3.9%) (Table 7.1). This

Table 7.1: Comparison between chorionic villus sampling and amniocentesis in two multi-centre trials (Medical Research Council European Trial of chorion villus sampling and Canadian Collaborative CVS-Amniocentesis Clinical Trial Group) and incidence of 'false positive' results due to confined placental mosaicism diagnosed in three multi-centre trials (Medical Research Council European Trial of chorion villus sampling, Canadian Collaborative CVS-Amniocentesis Clinical Trial Group and European Collaborative Study on mosaicism in chorionic villus sampling)

	CVS	Amniocentesis
Medical Research Council European Trial (1991)		
Abnormalities	5.2% (57/1102)	3.9% (39/1001)
Non-mosaic (confirmed*)	39 (17/18)	27 (12/12)
Mosaic (confirmed*)	18 (1/12)	12 (3/8)
Total False Positives	40.0% (12/30)	25% (5/20)
Confined Placental Mosaicism (% of false positives)	91.7% (11/12)	62.5% (5/8)
Canadian Collaborative CVS-Amniocentesis Clinical Trial (1989)		
Abnormalities	4.6% (43/933)	2.4% (22/932)
Confirmed*Abnormalities	55.8% (24/43)	86.4% (19/22)
Total False Positives	44.2% (19/43)	13.6% (3/22)
European Collaborative Study (1989)		
Mosaic	1.2% (141/11855)	
Confirmed*Mosaic	2.2% (3/141)	
Confined Placental Mosaicism (% of mosaics)	97.9% (138/141)	

* Confirmed means that the abnormality was also present in the fetus.

trend was also reported in the Canadian Collaborative CVS-Amniocentesis clinical trial (1989), where the abnormalities detected by CVS were 4.6% compared to 2.4% detected by amniocentesis. The greater proportion of abnormal diagnoses seen in the CVS groups of both of these studies was because the CVS had more cases of false positive diagnoses (diagnosis not confirmed in the fetus). Among the abnormal karyotypes, the incidence of false positive diagnoses was 40.0% and 44.2% in the Medical Research Council European Trial of chorion villus sampling (1991) and the Canadian Collaborative CVS-Amniocentesis Clinical Trial Group (1989) respectively. This compares with 25% and 13.6% of false positives in the amniocentesis groups of the MRC and the Canadian trials (Table 7.1). A large proportion of the false positive diagnoses reported in these studies are due to confined placental mosaicism. Of the 12 cases of confined placental mosaicism reported in the MRC trial, one was polyploid/diploid mosaic, while three out of 17 and 10 out of 141 (one triploid and 9 tetraploid), in the Canadian trial and European trials respectively, were polyploid/diploid mosaic.

Confined placental mosaicism is clearly a great problem when assessing CV samples. There is, however, an added complication when the mosaicism involves tetraploidy, not least that during cytogenetic analysis, the presence of tetraploid chromosome spreads are often regarded as cultural artefacts and ignored. Tetraploid cells are also known to exist naturally in the cytotrophoblastic layer of the villi (Wakuda, and Yoshida, 1992) and in amniotic fluid cells (West *et al,* 1989). Teshima *et al* (1992), reported that a low proportion of tetraploid cells was detected in 23 of 1019 CVS and 20 of 968 amniotic fluid samples, although in all 11 cases normal infants were born.

Another study (Kalousek *et al*, 1991) reported three cases of confined placental mosaicism where the abnormal cell line was tetraploid. In two of these cases, the

mosaicism was also apparent in term villi, but little or no tetraploid cells (<2%) were found either in the amnion or cord blood. No confined mosaicism was reported either in the term villi or the fetus of the third case. These data suggest that tetraploid cells may be present in the trophoblast of a conceptus, but absent from the fetus and amnion. It is interesting to draw parallels between these findings and the data gathered from tetraploid↔diploid chimaeras, discussed in Chapter 5.4. The apparent distribution of tetraploid cells seems similar in both the human and chimaeric mouse cases, in that the tetraploid cells are predominantly found in the trophoblast and are excluded from the fetus. There was good contribution of tetraploid cells to the yolk sac endoderm and parietal endoderm of the mouse conceptuses. Unfortunately the parietal endoderm is a structure found in rodents and not in humans, so the comparison cannot be extended in that direction. On the other hand, the yolk sac does exist in human conceptuses, although it is a small structure which degenerates towards term. It would be interesting, however, to study the yolk sacs of early abortuses. It is also interesting to link in both human and mouse uniform tetraploid conceptuses which very often amount to no more than an empty gestational sac with no evidence of fetal tissue.

7.3 FURTHER EXPERIMENTS

One or two questions which arose during the work described in this thesis remained unresolved, mainly due to lack of time. There are several experiments I would have liked to have completed to clarify these points before finishing the project.

The first unresolved point was whether, in tetraploid↔diploid chimaeras, the tetraploid cells were being excluded from the inner cell mass merely due to their size (see Chapter 5.3). The distribution of tetraploid cells in the tissues of the $12^1/_2$ day chimaeras analysed was fairly similar to that of cells derived from the 4-cell embryos in 4-cell↔8-cell chimaeras analysed at the same age. This result does seem to imply that selection or exclusion pressures are acting upon the size of the tetraploid cells, and

not upon their genetic abnormality. The production of 4-cell↔8-cell chimaeras was not an ideal control, however, since, to obtain 4-cell embryos on the same day as 8-cell embryos to make chimaeras, the donor female mice had to be superovulated 16 hours after those used to produce 8-cell embryos. This means that the 4-cell embryos were developmentally retarded compared to the 8-cell embryos, and may have been excluded from the inner cell mass because of this rather than their cell size. One experiment which could have been used to clarify the situation was to aggregate diploid 4-cell embryos to tetraploid 4-cell embryos. With this combination, the tetraploid cells could not be excluded due to size. However, the same problem exists, that the diploid 4-cell embryos would be developmentally retarded with respect to the tetraploid embryos. In this case the developmental delay may have been advantageous, by forcing the tetraploid cells into the inner cell mass.

A second question which remained unanswered was: By what mechanism are tetraploid cells excluded from the fetus during development of tetraploid↔diploid chimaeras? A few alternatives are discussed in Chapter 5.4, but the analysis at $12^1/_2$days of gestation was insufficient to determine absolutely which mechanism, or combination of mechanisms was in play. The alternatives discussed were; (i) abnormal cells are usually excluded from the inner cell mass, (ii) abnormal cells are usually excluded from the primitive ectoderm derivatives, (iii) tetraploid cells can contribute to three lineages early in development, but are selected against in the fetus. A way of elucidating which mechanism was responsible for the pattern of tetraploid cell distribution in tetraploid↔diploid chimaera, would have been to look at chimaeric conceptuses earlier in development, say at $7^1/_2$ days of gestation. An ideal way of studying the earlier conceptuses would have been by *in situ* hybridization. If signal was seen uniformly through the entire conceptus after hybridization, it is likely that tetraploid cells are excluded from the primitive ectoderm derivatives later in development, by a selection event. On the other hand, at $7^1/_2$ days, hybridization

signal confined to the primitive endoderm and trophectoderm derivatives would suggest that an allocation event earlier in development had excluded tetraploid cells from the primitive ectoderm. A few preliminary experiments were carried out at $7^1/_2$ days of gestation, but failed to show the presence of tetraploid cells by *in situ* hybridization. This may have merely been bad luck and none of the conceptuses were chimaeric, or, more likely there was no transgene to hybridize to since some of the males used were either hemizygous for or lacked the transgene when they should have been homozygous (see Chapter 5.3).

The failure of the Lac-Z transgenic to meet the requirements of the analysis of chimaeras at $12^1/_2$ days of gestation was disappointing but it is possible that in the very near future a more suitable construct and promoter will be developed to study mid-gestation conceptuses. I feel that the possibility of using a cytoplasmic cell marker in conjunction with DNA *in situ* hybridization would greatly aid the study of abnormal cells in histological sections, as it would be possible to identify both cell populations in a chimaera. A transgenic cellular marker would also be a useful way of studying wholemount conceptuses to try and determine the extent of cell mixing in the embryo as a whole.

The work described in this thesis has yielded interesting data on the fate of tetraploid cells in tetraploid↔diploid chimaeras, and on reliable methods of both producing and analysing tetraploid mouse embryos. Hopefully the data will be useful in the study of the clinical aspects of tetraploidy.

APPENDICES

I. EMBRYO CULTURE MEDIA

STOCK SOLUTIONS

Stock A (10x)	1M NaCl
	0.05M KCl
	1.2M KH_2PO_4
	0.01M $MgSO_4.7H_2O$
	0.23M Na pyruvate (60% solution)
	5.5mM Glucose
	10^5 units penicillin
	3.75 x 10^4 units streptomycin
Stock B (10x)	0.25M $NaHCO_3$
	0.01g phenol red
Stock C (100x)	0.33M Na pyruvate
Stock D (100x)	0.17M Ca $Cl_2.2H_20$
Stock E (10x)	0.25M HEPES
	0.01g phenol red

Adjusted to pH to 7.4 with 5M NaOH before making up to 100ml.

100ml of stocks A, B and E and 10ml of C and D were made. All stocks were filter sterilised and stored at 4°C. Stocks A, D and E were kept for 3 months and B and C changed every two weeks.

M16 CULTURE MEDIUM
(modified Kreb's-Ringer bicarbonate solution; Whitingham, 1971)

Stock	A	1.0ml
	B	1.0
	C	0.1
	D	0.1
Double distilled H_2O		7.8
BSA		4mg/ml

M2 HOLDING MEDIUM
(HEPES buffered modified Kreb's-Ringer solution; Quinn *et al*, 1982)

Stock	A	1.0ml
	B	0.16
	C	0.1
	D	0.1
	E	0.84
Double distilled H_2O		7.8
BSA		4mg/ml

Filter sterilised before use.

II. SUPEROVULATION

REAGENTS

PMSG Stock solution of 50 units/ml. Dosage : 5u per mouse (Gestyl, Organon)

hCG Stock solution of 50 units/ml. Dosage : 5u per mouse (in house standard, Reproductive Endocrinology, University of Edinburgh)

METHOD

Female mice were superovulated by injection of pregnant mare's serum gonadotrophin (PMSG), followed by human chorionic gonadotrophin (hCG) 48hr later (Hogan *et al*, 1986). The females were mated to males of the required strain, and checked for vaginal plugs the following morning. The plug date is equivalent to day $^1/_2$. Ovulation normally occurs 10-13 hr after hCG injection, and to obtain the best embryo yield, it is important to administer the hCG before the release of endogenous lutenizing hormone (LH). LH release is regulated by the the diurnal dark/light cycle and is released 15-20hr after the midpoint of the second dark period following PMSG administration (Hetherington, 1987). The animals were housed in a room with a light period between 5am and 7pm. For the collection of 8-cell embryos at $3^1/_2$ days, both PMSG and hCG were injected at midday, and for two-cell embryos at $1^1/_2$ days to undergo electrofusion, the hCG was administered at 4.30pm. This delay was to insure that the embryos were not in the process of dividing during the electrofusion procedure. For the production of 4-cell embryos, both the PMSG and hCG injections lagged the corresponding injection to produce 8-cell embryos by 16hr. The injections were administered at 5pm for 8-cell embryos and 9am the following morning for 4-cell, so that ovulation occurred at 3-6am and 7-10pm respectively. Injections were delayed by 24hr for the collection of 2-cell embryos. Donor females were sacrificed by cervical dislocation and either the oviducts or uterus were flushed out using M2 handling medium (see appendix I) and a fine syringe needle (Cooper's Needle Works,

Birmingham) (Pratt, 1987). Embryos at the two-cell and 4-cell stages were found in the oviducts, but by the 8-cell stage a proportion of embryos had moved down into into the uterus.

III. CHIMAERA PRODUCTION

REAGENTS

Acid Tyrode's solution

0.14M NaCl

2.6mM KCl

1.4mM $CaCl_2$

0.5mM $MgCl_2.6H_2O$

0.2mM$NaH_2PO_4.H_2O$

5.5mM Glucose

0.12M $NaHCO_3$

0.4% polyvinyl pyrrolidone

Adjusted to pH 2.5 with 5M HCl. Filter sterilised and stored at 4°C.

METHODS

Chimaeras were generated by aggregating pairs of embryos at the required stage of development [Tetraploid↔diploid, diploid↔diploid (2-cell↔8-cell, 4-cell↔8-cell or 8-cell↔8-cell)]. The zona pellucidae was removed from the embryos using acid Tyrode's solution, pH 2.5 (Nicholson *et al.*, 1975) either at 37°C or room temperature. Aggregation chimaeras were produced by standard procedures involving aggregation of pairs of zona-free embryos (Tarkowski, 1961; Mintz, 1962; McLaren, 1976). Embryos were transferred in pairs to drops of culture media under paraffin oil (Boots) in bacteriological grade petri dishes. Firstly, each pair was pushed together in a drop of M2+PHA [1 part phytohaemagglutinin (M form, GIBCO 670-0576) plus 19 parts M2 medium] to aid adhesion (Mintz *et al*, 1973; Pratt, 1987), incubated for 2 minutes at 37°C in 5% CO_2 in air, then rinsed in a drop of M2 (see appendix I). The aggregated embryos were then transferred drops of M16 medium under paraffin oil, in

a dish that had been equilibrated overnight at 37°C in 5% CO_2 in air. Any pairs of embryos that had drifted apart were pushed together again and the aggregated pairs were cultured overnight. (Some developmental asynchrony is likely between the aggregated embryos because they were aggregated soon after collection, without checking the time of their previous cleavage division.)

IV. TRANSFER TO PSEUDOPREGNANT RECIPIENTS

REAGENTS

Anaesthetic

Hypnorm (0.315 mg/ml fentanyl citrate and 10mg/ml fluanisone; Janssen Pharmaceuticals)

Hypnovel (2mg/ml midazlam hydrochloride; Roche)

METHOD

Pseudopregnant Recipients

Females to be used as transfer recipients were either superovulated (see appendix II) or selected in oestrus (Champlin *et al*, 1973) and mated to vasectomised males. Plugs were checked the following morning, which was counted as day $^1/_2$ and the transfer was performed on day $2^1/_2$. The embryos were collected in a small amount of M2 handling medium (see appendix I) in a fine glass pipette, and were surgically transferred to the uterus, as close to the oviduct as possible, via a small incision made with a fine needle (McLaren and Michie, 1956). Pseudopregnant females were anaesthetised with 0.25 ml per 30 g body weight of a 1:1 v/v mixture of Hypnorm and Hypnovel .Both the Hypnorm and Hypnovel were diluted 1:1 v/v with sterile distilled water, before being mixed together. Pregnancies were timed according to the pseudopregnant female.

V. EMBRYO DISSECTIONS

REAGENTS

Trypsin/pancreatin Solution

0.5g trypsin (1000-2000 units/mg)

2.5g pancreatin (Sigma p1500)

100ml PBS

Filtered through Whatman's filter paper, then filter sterilised.

METHOD

The females were sacrificed by cervical dislocation at $12^1/_2$ days gestation and the conceptuses were dissected out into saline (Hogan et al, 1986). The conceptuses, fetuses and placentas were weighed, the crown rump length was measured under the dissecting microscope using a glass grid (R10, Graticules; pitch 0.1mm) and the hind limb morphology was assessed as a guide to developmental stage (McLaren and Buehr, 1990). The conceptuses were then dissected into their constituent tissues as described by Papaioannou and West (1981): placenta, trophoblast, Reichert's membrane, yolk sac endoderm, yolk sac mesoderm, amnion and fetus. The trophoblast and decidual tissue was dissected carefully away from Reichert's membrane in a layer. The whole yolk sac was separated into the endoderm and mesoderm layers by incubation in trypsin/pancreatin at 4°C for $2^1/_2$-3hr, followed by washing in M2 culture medium (Levak-Svajger and Skreb 1969). The membranes were stored at -20°C in 10 µl of 50% glycerol in water in microtitre plates. The fetus and placenta in were stored in 100µl of 50% glycerol in 1.5ml microtubes. Samples were lysed by freeze/thawing, with mechanical disruption of the fetus and placenta, in preparation for electrophoresis.

VI. ENZYME ELECTROPHORESIS

REAGENTS

Citric acid

EDTA

Fructose 6 phosphate (F6P)

Glucose 6 phosphate dehydrogenase (6-10units)(G6P)

Glycine

$MgCl_2.6H_2O$

NADP

Nitro blue tetrazolium (NBT)

Phenozine methosulphate (PMS)

Sodium citrate

Tris

Stock Solutions

F6P	20mg/ml
$MgCl_2$	0.2%
NADP	2.7mg/ml
NBT	2.7mg/ml
PMS	2.5mg/ml

Tris citric acid pH8

0.3M Tris

0.08M Citric acid

Electrophoresis Buffer

0.02M Tris

0.2M Glycine

Adjust to pH 8.1 and store for one month at 4°C.

Stain (1 plate)

Glycerol/MgCl2 (50:50)	1.5ml
Tris citrate	170µl
F6P	170µl
NBT	170µl
NADP	170µl
G6PD	10units
PMS	20µl

METHOD

Electrophoresis was carried out using electrophoresis tanks and Super Z-12 applicators obtained from Helena Laboratories and a Consort Bioblock Scientific power pack. Titan III cellulose acetate plates (Helena Laboratories) were soaked in electrophoresis buffer for 30min. The tank reservoirs were filled with electrophoresis buffer and Whatman filter paper was used to form wicks (Hebert, 1989). Samples were applied to blotted plates and run from anode to cathode at 200v for 60min, then stained for glucose phosphate isomerase (GPI-1) activity for up to 20min on a 37°C hotplate. After rinsing in water, the plates were fixed in 5% acetic acid for 5min then washed in distilled water for 15min.

Scanning densitometry was carried out on dry plates, without clearing in oil, using using a Helena Process-24 gel scanner to determine the relative proportions of GPI-1A and GPI-1B in the samples (West *et al*, 1986).

VII. WAX HISTOLOGY

FIXATION

Samples to be processed were contained in TissueTex cassettes (Miles Ltd., Slough) and fixed in 3:1 (ethanol:acetic acid) at 4°C, overnight or for a minimum of 6hr. at 4°C. After removal to 70% ethanol it was possible to store the samples prior to processing, for several weeks at 4°C, with several changes of 70% ethanol.

TISSUE PROCESSING

70% ethanol	over night (up to several weeks)
100% ethanol	2x 30min
Histo-Clear	3x 60min
Paraffin wax/Histo-Clear	60 min
Paraffin wax (vacuum)	60min

After processing the samples were embedded in paraffin wax (Miles Ltd., Slough) using a Reichert-Jung tissue embedding centre and stored at 4°C.

SECTIONING

Slides were washed in ethanol/HCl and dried. After dipping in 2% TESPA (3-amino propyl triethoxysilane)(Sigma) in acetone, and air drying, the slides were redipped in TESPA, then rinsed in acetone followed by distilled H_2O, and allowed to dry. Sections of the tissues were cut at using an 820 Spencer Microtome (American Optical Corporation), and floated from a water bath at 45°C onto the TESPA coated slides. The prepared slides were dried for at least 48 hr. at 37°C or 24 hr. at 42°C prior to further treatment.

VIII. *IN SITU* HYBRIDIZATION

REAGENTS

TE buffer

10mM Tris pH 7.5, 1mM EDTA

50x Denhardt

Bovine serum albumin	0.5g
Polyvinyl pyrrolidone	0.5g
Ficoll	0.5g
H_2O	50ml

20 x SSC

3M NaCl, 0.3M sodium citrate, pH 7-7.4

Buffer 1

0.1M Tris, 0.15M NaCl, pH7.5

DAB Buffer

50mM Tris, pH 7.3

Development Reagent

0.5mg/ml diamino benzidine (DAB) in DAB buffer, with 4µl/10ml of H_2O2 added just before use.

Harris's Haemotoxylin

Haemotoxylin (Gurr)	2.5g
Absolute alcohol	25ml
Potassium alum	50g
Distilled H_2O	500ml
Mercuric oxide	1.25g
Glacial acetic acid	20ml

The haemotoxylin was dissolved in the alcohol, and added to the alum previously dissolved in the warm distilled water. The mixture was rapidly brought to the boil in a 2l flask and the mercuric oxide added. The stain was rapidly cooled on ice and the acetic acid added when cold.

Eosin (Yellowish)

1% aqueous eosin (BDH) solution:1% alcohol eosin solution, 3:1

0.05% acetic acid added before use.

Acid Alcohol

70% ethanol:conc.HCl, 99:1

Scott's Tap Water

Potassium bicarbonate	2g
Magnesium sulphate	20g
Distilled water	1l

METHODS

Labelling the Probe

The plasmid pMßð2 was linearised by incubation at 37°C with *Eco*RI (1 units/µg) for 90min. A sample was run on a 0.8% agarose gel to confirm that restriction had taken place. Phenol/chloroform extraction was performed and the DNA was ethanol precipitated overnight at -20°C. The precipitate was dissolved in TE buffer and stored as a stock of 100ng/µl.The probe was denatured by boiling 1µg of DNA in distilled water in a total volume of 10µl, then removing to ice. The labelling reaction was carried out using reagents from the Boehringer Mannheim DIG DNA Labelling and Detection Kit.

Denatured probe	10µl
10x hexanucleotide	2µl
10x dNTP	2µl
sterile distilled H_2O	5µl

The reaction mixture was incubated at 37°C for 6hr with 2U/µl of Klenow enzyme (1µl), then stopped by the addition of 2µl of 0.2M EDTA pH 8. The labelled DNA was precipitated overnight at -20°C with cold ethanol and 2µl of 3M sodium acetate, then redissolved in 50µl of TE buffer to give a final stock concentration of 20 ng/µl.

Slide Pretreatment

Slides with sections of fixed tissues were pretreated to facilitate hybridization.

Histo-Clear	2x10min	
100% ethanol	2x5min	
3% H_2O_2/methanol	30min	
70% ethanol	2x5min	
PBS	2x10min	
1mMNaOH	3min	70°C
PBS	2x5min	4°C

Prehybridization

for 10 slides	*vol (µl)*	*final conc.*
50x Denhardt	80	5x
deionised formamide	360	45%
20x SSC	240	6x
sonicated salmon sperm DNA (10mg/ml)	40	500µg/ml
double distilled H_2O	80	

Samples were prehybridized for 15 min at 60°C using 50µl/slide of prehybridization mix with unsealed coverslips in a pre-heated humidified chamber.

Hybridization

for 10 slides	*vol (µl)*	*final conc.*
denatured probe	10	20ng/slide
sonicated salmon sperm DNA (10mg/ml)	20	500µg/ml
20% dextran sulphate	200	10%
20 x SSC	100	5x
double distilled H_2O	70	

Samples were hybridized overnight at 60°C using 40µl/slide of hybridization mix and hydrophobic coverslips (Gel-Bond) sealed with nail varnish in a humidified chamber.

Washes

The coverslips were removed and the slides were washed to remove non-specifically bound probe.

2 x SSC, 0.1% Triton-X	2x5min	
0.1 x SSC, 0.1% Triton-X	5min	
0.1 x SSC, 0.1% Triton-X	5min	50°C
5% BSA, 0.1 x SSC, 0.1% Triton-X	5min	

Visualisation

Buffer 1	5min

The stock concentration of the antibody was 150U/ml. A 1:100 dilution of this was made.in buffer 1 and 40µl was added to each slide under unsealed coverslips in a dark, humidified chamber. The slides were incubated for 30 min.

Buffer 1	2x10min
DAB buffer	3min

The slides were placed in a dark humidified chamber and flooded with development reagent for 40 min. The reaction was quenched in water. The slides were counter-stained with haemotoxylin and eosin.

Counter-staining

Haemotoxylin	2min
Rinse in water	
Acid alcohol	10s
Rinse in water	
Scott's tap water	30s
Rinse in water	
Eosin	10s
70% ethanol	20s
95% ethanol	20s
100% ethanol	20s
Histo-Clear	5min

After counter-staining and dehydration, the slides were mounted with Histo-Mount (National Diagnostics).

IX. ELECTROFUSION

REAGENTS

Non-electrolytic Electrofusion media

	50ml
0.3M Mannitol	2.733g
0.5% PVP	0.25
0.1mM HEPES	0.0012 (10x stock 1.2g/100ml, Add 100µl to 50ml)
phenol red	5mg

Make up to 50mls with sterile H_2O, pH to 7.3 with 0.1M NaOH and filter sterilise.

METHOD

The electrofusion apparatus consisted of a perspex disc with two platinum electrodes embedded into the upper surface 180µm apart. The disc rested inside a plastic petri dish. The electrodes were connected to a Digitimer High Voltage Stimulator via wire leads.

To perform electrofusion, the chamber was filled with electrofusion medium. After washing the 2-cell embryos twice in drops of electrofusion medium, they were positioned between the platinum wires using a dissecting microscope (Wild, Heerbruug). Two to three 150v square pulses of 50µs duration were applied, then the embryos were washed through four drops of M16 culture medium (see appendix I) under oil. After 15-30 min, embryos which had undergone fusion of the two blastomeres into one cell were removed to separate drops, and the unfused embryos were subjected to a second treatment of electrofusion. Approximately one third of treated embryos fused on each round of fusion. Fused embryos were incubated overnight at 37°C, before further manipulation.

X. LAC Z TRANSGENE

REAGENTS

0.1M phosphate buffer pH 7.3

0.1M disodium hydrogen orthophosphate:0.1M sodium dihydrogenorthophosphate

21 volumes:4 volumes

Gluteraldehyde Fixative

0.2% gluteraldehyde

0.1M phosphate buffer pH 7.3

2mM $MgCl_2$

5mM EGTA pH 8

X-Gal Wash

0.1M phosphate buffer pH 7.3

2mM MgCl

0.1 sodium desoxycholate

0.02% NP40

0.05% BSA

X-Gal

25mg X-Gal (5-bromo 4-chloro 3-indolyl ß-D galactopyranoside) dissolved in 0.5ml dimethyl formamide

X-Gal Stain

25ml Wash plus: 6mg spermidine

41mg $K_3Fe(CN)_6$

52.5mg $K_4Fe(CN)_6$

0.4ml 0.085% NaCl

100µl of X-Gal was added to 5ml of stain and filter sterilised (1mg/ml X-Gal and 5mM Fe^{3+}/Fe^{2+}).

METHOD

The embryos were dissected from the females at the required age into cold PBS in a micro-titre plate on ice, then transferred to 0.2% gluteraldyhyde and fixed for 5-60min (according to size) at 4°C. The embryos were then given three washes of 20min in X-Gal wash at room temperature, before being transferred to the stain. The staining took place overnight at 37°C, in the dark. The ear punches were treated similarly, being fixed for 5min, and washed twice. 75µl of each reagent was used per microtitre plate well. After staining the samples were fixed in 3:1 (ethanol:acetic acid) for histology (see appendix VII).

XI. MOUSE STRAINS

Inbred Strains

C57BL	
CBA/Ca	(obtained from J. Ansell, ICAPB, Edinburgh University)
BALB/c	(obtained from J. Verth, Medical Microbiology, Edinburgh University)

Congenic Strains

BC	C57BL/Ola.AKR-*c* *Gpi-1s*^{a}/Ws
CC	C57BL/Ola-*c* *Gpi-1s*^{c}/Ws
CALB	BALB/c-*Gpi-1s*^{c}

Transgenic Strains

CLO	Strain 83 (Lo, 1986)
CMA	Tg/Tg (derived by intercrossing Strain 83 and BC)
Tg(Act-lac Z)-1	on PO background (Beddington *et al*, 1989)
ACTA	*Tg(Act-lac Z)-1* crossed to BC

F_1 Hybrid Stocks

AF_1	(BCxBALB/c)F_1
BF_1	(C57BLxCBA/Ca)F_1
CF_1	(CCxCALB)F_1

XII. STATISTICAL ANALYSIS

The statistical tests were performed on an Apple Macintosh computer, using the "Multistat" statistical package (Biosoft, Cambridge).

For normally distributed data, the following statistical tests were used:

The mean of a population, $\bar{x}$, given by

$$\bar{x}=\Sigma x/n$$

The estimate of variance, s^2, given by

$$s^2=\Sigma(x-\bar{x})^2/(n-1)$$

The standard deviation, SD, given as the square root of the variance

$$SD=\sqrt{\{\Sigma(x-\bar{x})^2/(n-1)\}}$$

The estimate of the standard error of the mean, SEM, given as

$$SEM=SD/\sqrt{n}$$

The Student's t test was used to compare the means of two normally distributed samples, to test the null hypothesis that there is no difference between the means of two samples. The value of t is given by

$$t=\frac{(\bar{x}_1-\bar{x}_2)}{\sqrt{s^2(1/n_1+1/n_2)}}$$

where $s^2=\{\Sigma(x_1-\bar{x}_1)^2+\Sigma(x_2-\bar{x}_2)^2\}/(n_1+n_2-2)$

The coefficient of variation was used to express the ratio of the standard deviation to the mean, and is given by

$$=SDx100/\bar{x}$$

The following non-parametric tests were applied to data which was not normally distributed:

The Mann-Whitney U Test was used to compare the medians of two sets of data, to test the null hypothesis that there is no difference between the medians of two samples. The U values are given by

$$U_1=N_1N_2+{}^1/_2N_2(N_2+1)-R_2$$

and $$U_2=N_2N_1+{}^1/_2N_1(N_1+1)-R_1$$

where the smaller value of U is the found value

where R= sum of the ranks

N= number samples

The Kruskal Wallis one-way analysis of variance by ranks was used to compare the medians of two or more sets of data. The value of KW is given by

$$KW={}^{12}/_{N(N+1)}\sum_{j=1}^{k} n_j\,(\bar{R_j}-\bar{R})^2$$

where k= number of samples

n_j= number of cases in the jth sample

N= number of cases in the combined sample (the sum of the n_j's)

R_j= sum of the ranks in the jth sample

$\bar{R_j}$= average of the ranks in the jth sample

$\bar{R}$= $(N+1)/2$=the average of the ranks in the combined sample (grand mean)

The Spearman rank-order correlation was used to investigate the association between sets of data. The coefficient of correlation, r_s, is given as

$$r_s=1-\ (6\sum_{i=1}^{N} d_i^2)/N^3-N$$

where N= number of samples

d_i= X_i-Yi= an indication of the disparity between the two sets of rankings

The χ^2 test was used to test the null hypotheses that there is no difference between the distributions of the ratios sets of data. The value for χ^2 is given as

$$\chi^2=\Sigma(O-E)^2/E$$

where O is the observed value and E is the expected value. Where the expected number was less than 5, Yates correction factor was applied, and χ^2 is then given, for a 2x2 contingency table withe frequencies of a, b, c and d, as

$$\chi^2=\frac{n\{|ad-bc|-1/2n\}^2}{(a+b)(c+d)(a+c)(b+d)}$$

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Electrofusion of mouse embryos results in uniform tetraploidy and not tetraploid/diploid mosaicism

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Summary

Some previous attempts to produce tetraploids experimentally have resulted in a proportion of treated embryos becoming 2n/4n mosaics at a frequency which may be as high as 20%, when using cytochalasin B as a fusigenic stimulus and cytogenetic techniques to identify putative tetraploid embryos. To investigate the possible occurrence of 4n/2n mosaicism, tetraploid embryos were produced by electrofusion, a process which allows adjacent blastomeres at the 2-cell stage to fuse following exposure to electric field pulses. Embryos used for electrofusion were hemizygous for a transgene consisting of approximately 1000 copies of the mouse β-globin gene. After *in situ* hybridization, one hybridization signal is expected per diploid genome. Tetraploid cells in 7·5-, 8·5-, 9·5- and 10·5-day-old conceptuses were distinguished from diploid cells by performing *in situ* hybridization on histological sections. The frequency of nuclei with two hybridization signals in the 'hemizygous' tetraploid embryos was compared to diploid embryos which were either hemizygous or homozygous for the β-globin transgene. Comparison of the frequency of nuclei with two hybridization signals between tissues of 'hemizygous' tetraploid conceptuses and homozygous diploid conceptuses showed no significant difference, which implies that the tissues in the tetraploid conceptuses were uniformly tetraploid. No evidence was found to suggest that electrofusion results in 2n/4n mosaicism.

1. Introduction

The phenomenon of polyploidy is a well documented natural occurrence in plants, invertebrates and some vertebrates (Beatty & Fischberg, 1949), which are able to tolerate and compensate for polyploidy throughout the whole organism, but it appears to be a lethal or sublethal condition in mammals, including man. Tetraploidy frequently occurs in amniotic fluid cells and some instances of partial hydatidiform mole (Surti *et al.* 1986), as well as in other tissues (Wilson *et al.* 1988).

Although somewhere in the region of 1·3% of first trimester abortuses are tetraploid (Scarbrough *et al.* 1984), very few cases of liveborn infants have been reported (Golbus *et al.* 1976; Pitt *et al.* 1981; Scarbrough *et al.* 1984; Shiono *et al.* 1988; Pajares *et al.* 1990). Several theories have been proposed to describe the events which may give rise to a tetraploid conceptus; trispermic (3 haploid sperm) or dispermic (1 haploid and 1 diploid sperm) fertilization of a haploid ovum (Sheppard *et al.* 1982; Surti *et al.* 1986). Alternatively fertilization by either two haploid or one diploid sperm after suppression of first or second maternal meiotic division (Scarbrough *et al.* 1984). The sex chromosome complement of reported tetraploid cases is generally either XXYY or XXXX, and this fact along with information obtained by performing chromosome banding techniques (Kajii & Niikawa, 1977) on abortuses and their parents strongly suggests that tetraploidy principally arises due to suppression of cell division at the first cleavage division of the zygote (Pitt *et al.* 1981). However, the majority of human liveborn tetraploid cases reported have been mosaics with a tetraploid and another cell line, usually 4n/2n (Scarbrough *et al.* 1984; Warburton *et al.* 1991). Mosaicism may arise due to failure of cytoplasmic cleavage at a later mitotic division. In some cases 4n/2n mosaic individuals are only diagnosed after presenting with a well defined clinical

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syndrome such as Ullrich–Turner or Noohan (Wilson *et al.* 1988).

Several methods have been devised to induce tetraploidy experimentally in mammalian embryos in order to develop a suitable animal model to study the behaviour of polyploid cells within the embryo as a whole. Three techniques have been described to date which involve: (1) the suppression of cell division using colchicine (Edwards, 1958) or cytochalasin B (Snow, 1973, 1975, 1976; Tarkowski *et al.* 1977), (2) microsurgical transfer of nuclei from morula cells to fertilized one cell eggs (Modlinski, 1978) or (3) blastomere fusion at the 2-cell stage. Previously the production of tetraploids by cell fusion involved either virally-assisted blastomere fusion employing inactivated Sendai virus (Graham, 1971; O'Neill *et al.* 1990), or aggregation of 4-cell blastomeres into pairs using phytohaemagglutinin (PHA), then treating with 45% w/v polyethylene glycol (PEG) (Eglitis, 1980). More recently, the technique of electrofusion has been developed, which causes degradation of the cell membrane following exposure to electric field pulses, allowing adjacent cells to fuse (Kubiak & Tarkowski, 1985; Ozil & Modlinski, 1986; Kurischko & Berg, 1986; Kaufman & Webb, 1990; Petzoldt, 1991). The advantages of using electrofusion over other methods to produce tetraploids are that, unlike using cytochalasin B, it is not necessary for the cells to be at any particular stage in the cell cycle, nor are the embryos exposed to toxic chemicals. The time of exposure to the fusigenic stimulus is also very short, in the range of milliseconds.

Up to 20% of tetraploid embryos produced by the suppression of the second cleavage division may undergo reversion to 2n/4n mosaics, when using cytochalasin B (Tarkowski *et al.* 1977). Identification of putative tetraploids in the past has relied on cytogenetic techniques performed on embryonic and extraembryonic tissues. This method can only be applied to dividing cells, which constitute a small proportion of the total number of cells in the conceptus, so that it is difficult to say whether the sample is typical of the particular tissue studied, or of the embryo as a whole. The ideal solution to the problem would be a cellular marker through which it was possible to distinguish between diploid and tetraploid cells in histological sections, thereby determining the exact location and distribution of tetraploid cells over the whole conceptus. One such system employs a transgene, consisting of approximately 1000 copies of the mouse β-globin gene, which has been inserted into the mouse genome and may be detected by *in situ* hybridization (Lo, 1983).

By producing tetraploids from embryos carrying the transgene, tetraploid cells may be distinguished from diploid cells by the number of hybridization signals present in the nucleus after performing *in situ* hybridization. In this paper, the technique of *in situ* hybridization is used to investigate tetraploid conceptuses produced by electrofusion. By determinin whether, and what percentage of, fused embryos ar prone to mosaicism, it will be possible to decid whether the process of electrofusion is consistent. Th technique will also facilitate estimation of the pro portion of diploid:tetraploid cells, as well as thei distribution within the embryo.

2. MATERIALS AND METHODS

(i) *Embryo collection*

Female mice (see below) were superovulated b injection of pregnant mare's serum gonadotrophi (PMSG), followed by human chorionic gonadotrc phin (hCG) 2 days later (Hogan *et al.* 1986). Th females were mated to males of the required strai and checked for vaginal plugs the following morning The plug date is equivalent to day 0·5 p.c. For th collection of morula or blastocyst stage embryos at 3· days, both PMSG and hCG were injected at midda and for two cell stage embryos at 1·5 days, the hC was administered at 16·30 h. This delay in hC injection was to ensure that the embryos were not i the process of dividing during the electrofusio procedure.

(ii) *Tetraploid production*

Embryos for electrofusion were obtained by crossir (C57BL/Ws × CBA/Ca) F_1 females to strain 83 mal (Lo, 1983), which are homozygous for a transge comprising approximately 1000 copies of the mou β-globin gene. Two cell stage embryos were flushe from the oviducts and electrofusion was employed, described by Kaufman & Webb (1990) to genera tetraploid embryos. Fused reconstituted '1-cell stag embryos were transferred to the oviducts of pseud pregnant F_1 females on the first day of pseud pregnancy (on the morning of the presence of vaginal plug after mating to a vasectomized male).

(iii) *Diploid controls*

Two series of diploid controls, homozygous a hemizygous, were generated to estimate the extent developmental retardation in the tetraploid embry and to compare the percentages of labelled nuclei the tetraploid embryos with those in homozygo diploid and hemizygous diploid embryos. Hom zygous diploid controls were generated by mati strain 83 females to strain 83 males, resulting diploid embryos with two copies of the β-glob transgene (*Tg/Tg*). F_1 females mated to strain males produced hemizygous (single copy) diplc embryos (*Tg/−*). Morula or blastocyst cell sta embryos were flushed from the oviduct or uter

Pratt, 1987) and the embryos were transferred mmediately to the uterus of pseudopregnant F_1 'emales.

iv) *Histology*

'utative tetraploid conceptuses were isolated at 7·5, ·5, 9·5 and 10·5 days of gestation, diploids at 6·5, 7·5 nd 8·5 days, and fixed in 3:1 (ethanol:acetic acid) at °C for a minimum of 6 h. After transfer to 70% thanol it was possible to store the embryos for everal weeks at 4 °C, prior to processing, with several hanges of 70% ethanol. The processing procedure nvolved dehydration through ethanol, followed by Histo-Clear (National Diagnostic), then immersion in 50:50 Histo-Clear:paraffin wax mixture, before araffin wax, under vacuum. After processing, the amples were embedded in wax and stored at 4 °C. erial sections of the tetraploid and diploid conceptuses were cut at 7 and 5 μm respectively (see esults), and floated onto TESPA (3-amino propyl riethoxysilane) coated slides.

v) In situ *hybridization*

he DNA probe, pM$\beta\delta$2, used to detect the β-globin equences in the strain 83-derived cells of the etraploids was provided by Dr John Ansell, Institute f Cell, Animal and Population Biology, University of dinburgh. The probe plasmid, pM$\beta\delta$2, is derived om the plasmid pMJ, which is inserted into the ansgenic strain 83 mice (Lo, 1983). The plasmid was nearised using the restriction enzyme *Eco*R I and abelled by random primed incorporation of igoxigenin-labelled deoxyuridine-triphosphate, using Nonradioactive DNA Labelling and Detection Kit Boehringer, Mannheim). Routinely 1 μg was labelled sing the kit reagents to produce a stock concentration f 20 ng/μl (Keighren & West, 1992).

Hybridization procedures were modified from those f Morris *et al.* (1990) and Dr Reinald Fundele ersonal communication). Before performing *in situ* ybridization, the slides were dewaxed by immersion n Histo-Clear, rehydrated through a series of graded cohols, then washed in PBS. Endogenous peroxidase ctivity was inhibited by immersion for 30 min in 3% ydrogen peroxide in methanol, and the DNA present n the sections was denatured using 1 mM-NaOH at) °C, followed by PBS at 4 °C. The probe was enatured by boiling for 10 min, then removing to ice r a further 5 min. After prehybridization for 15 min . 60 °C in a humid box, hybridization took place nder sealed hydrophobic coverslips (Gel Bond), vernight at 60 °C, using the β-globin probe at a ncentration of 20 ng/slide.

After hybridization the slides were washed in a ries of solutions of SSC of increasing stringency with 0·1% Triton-X detergent added, and then with 5% BSA added to block non-specific conjugation of the antibody used to detect the digoxygenin-labelled probe. The antibody used (Boehringer, Mannheim) was an anti-digoxygenin antibody from sheep; Fab fragments conjugated with horseradish peroxidase (HRP). After binding of the anti-digoxygenin antibody the slides were washed and flooded with the development reagent which contained the peroxidase substrate diaminobenzidine. The product of the reaction is a brown, water and ethanol insoluble precipitate, which can be viewed under bright field, phase contrast or dark-field light microscopy. The sections were counterstained with haematoxylin and eosin, dehydrated through graded alcohols, followed by immersion in Histo-Clear, before being mounted with Histo-Mount (National Diagnostic).

3. Results

(i) *Section thickness*

Histological sections were analysed in preference to cell spreads so that it was possible to test whether there were tissue specific differences in the proportions of the diploid and tetraploid cells within the conceptus. The section thickness has a crucial bearing on the percentage of nuclei scored as positive for the hybridization signal, as well as the proportions scored as having one or two signals. If sections are too thin compared to the nuclear diameter, some cells will lack a section of nucleus, creating a higher incidence of false negative results. If the sections are too thick, overlapping of nuclei occurs, making scoring difficult (Thomson & Solter, 1988). Tetraploid sections were cut at 7 μm, but since the diploid nuclei are expected to be smaller than tetraploid nuclei, thinner sections were used to try and ensure that a similar proportion of the nucleus was present in the sections. The ratio of nuclear diameters (4n:2n) was estimated in two ways. Firstly, assuming that the tetraploid:diploid nuclear volume ratio is 2:1 (Henery & Kaufman, 1992), the corresponding ratio for nuclear diameters can be calculated as 1·26:1 (Epstein, 1986), given that the volume of a sphere is $4/3\ \pi r^3$. Thus, to ensure that a comparable proportion of the nucleus was included in histological sections, the section thickness for tetraploid samples should be 1·26× greater than for diploid tissue. For tetraploid samples cut at 7 μm, the appropriate thickness for diploid sections would be 5·56 μm. A second calculation was made, based on observed nuclear diameters, in matched material. The nuclear length and breadth was measured in 8 areas of the embryo, 4 areas of parietal endoderm, 4 areas of ectoplacental cone and 4 areas of trophoblast of a 7·5-day tetraploid conceptus and a 6·5-day hemizygous diploid conceptus. The average of the two values was calculated for each cell to estimate the nuclear diameter and the mean for each tissue calculated. The mean

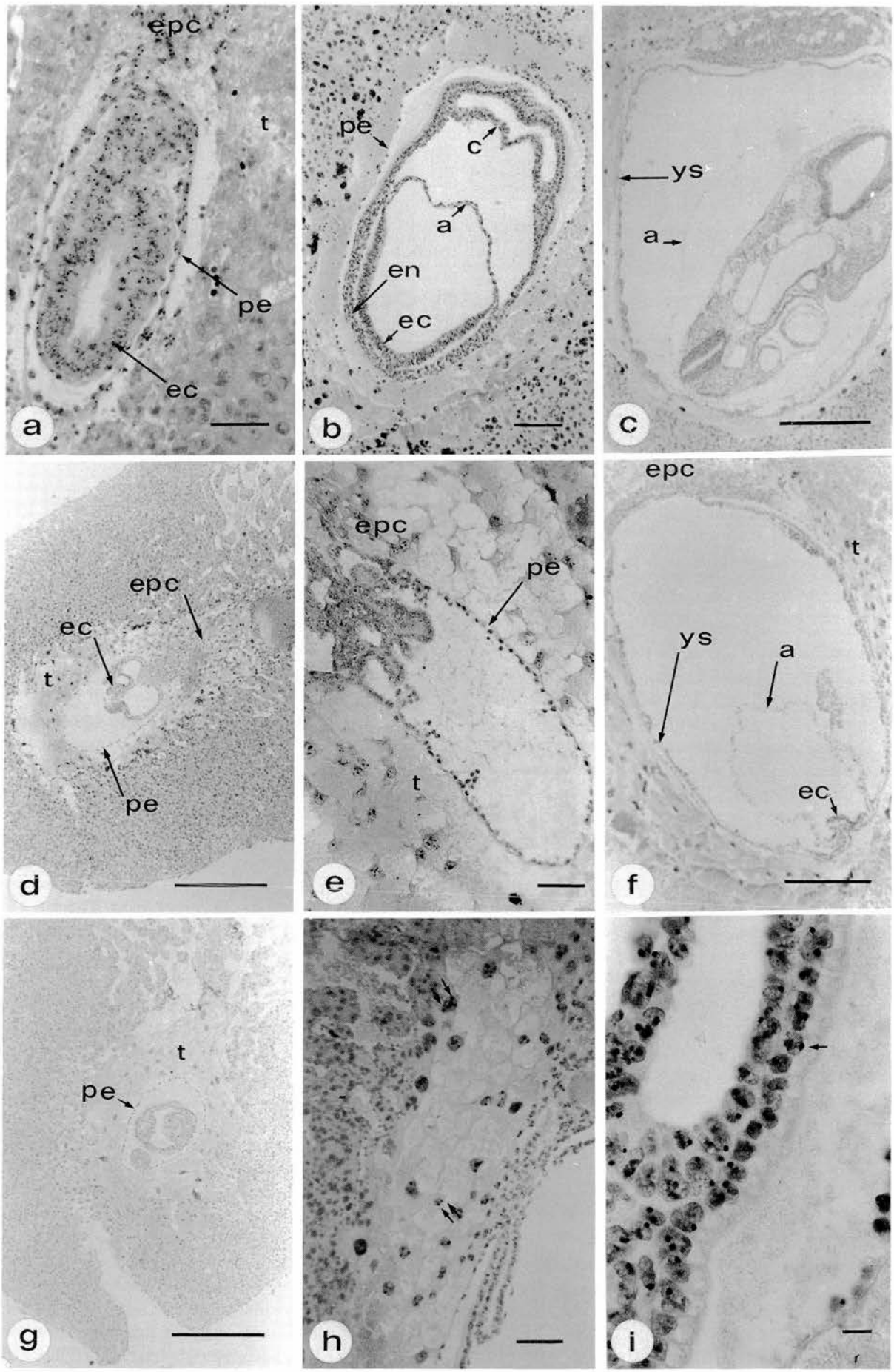

Fig. 1. For legend see opposite.

Table 1. *Comparison of mean nuclear diameter of tetraploid and diploid cells*

Tissue	No. of nuclei	Mean (±S.E.) nuclear diameter (μm)		Mean ratio 4n/2n
		Tetraploid	Diploid	
Embryo	8	11·64±0·58	7·10±0·46	1·64
Parietal endoderm	4	10·02±1·08	6·35±0·36	1·58
Ectoplacental cone	4	10·88±2·12	7·70±0·68	1·41
Trophoblast	4	11·96±0·71	7·70±0·26	1·55
Total mean ratio (all tissues)				1·56

Table 2. *The proportion and percentage of hybridization signals in various tissues of tetraploid embryos of different developmental ages detected by* in situ *hybridization*

Tissue	Age (days)	No. of embryos scored	Total nuclei scored	Nuclei with 0 signal	Nuclei with 1 signal	Nuclei with 2 signals	% positive nuclei with 2 signals
Tetraploid embryos, hemizygous (7 μm sections)							
Embryo	7·5	3	86	23	23	40	63·5
	8·5	4	101	19	34	48	58·5
	9·5	4	133	29	56	48	46·1
	10·5	3	90	26	36	28	43·7
Parietal endoderm	7·5	3	97	18	32	47	59·5
	8·5	4	249	53	74	122	62·2
	9·5	4	482	107	145	230	61·3
	10·5	3	163	41	70	52	42·6
Ectoplacental cone	7·5	3	46	11	11	24	68·6
	8·5	2	25	3	11	11	50·0
	9·5	2	24	5	8	11	57·9
	10·5	2	24	4	9	11	55·0
Yolk sac (whole)	9·5	2	502	111	180	211	54·0
Yolk sac endoderm*	9·5	1	24	2	14	8	36·4
Yolk sac mesoderm*	9·5	1	18	2	5	11	68·7
Trophoblast	7·5	1	64	†	33	31	48·4
	8·5	1	9	†	5	4	44·4

* From same embryo.
† All trophoblast cells scored were positive (hybridization signal distinguished them from maternal decidua cells).

ratio between tetraploid and diploid nuclear diameters was calculated for each tissue along with the ratio for all tissues (Table 1). The mean ratio of 1·56, was used to calculate a section thickness for the diploid embryos of 4·48 μm (equivalent to 7 μm for the tetraploid). The actual section thickness used for the diploid embryos was 5 μm, which is close to both the theoretical (5·56 μm) and calculated (4·48 μm) values.

(ii) *Morphology*

The development of the tetraploid embryos was retarded with respect to the diploid control conceptuses which agrees with previous studies of tetraploid development (Snow, 1975; Tarkowski *et al.* 1977). Fig. 1*a–c* (6·5, 7·5 and 8·5 days old respectively) shows histological sections of diploid embryos

Fig. 1. Histological sections of diploid (*a–c*, *i*) and tetraploid (*d–h*) mouse conceptuses, after *in situ* hybridization to detect the transgenic β-globin sequence (*Tg*), showing poor embryonic development of tetraploids. Bar is 10 μm in (*i*) 50 μm in (*a*), 100 μm in (*b*, *e*, *h*) and 500 μm in (*c*, *d*, *f*, *g*). (*a*) Normal 6·5-day homozygous, *Tg/Tg* diploid; (*b*) normal 7·5-day heterozygous, *Tg/−* diploid; (*c*) normal 8·5-day homozygous, *Tg/Tg* diploid. (*d–g*) 'Hemizygous', *Tg/Tg/−/−* tetraploid conceptuses at (*d*) 7·5 days, (*e*) 8·5 days, (*f*) 9·5 days and (*g*) 10·5 days. (*h*) Part of a 10·5-day 'hemizygous', *Tg/Tg/−/−* tetraploid showing trophoblast giant cells with two hybridization signals (arrows). (*i*) High power of part of normal 7·5-day heterozygous, *Tg/−* diploid conceptus (*b*), showing one hybridization signal in most nuclei.
Abbreviations: a, amnion; c, chorion; ec, embryonic ectoderm; en, embryonic endoderm with subjacent mesoderm; epc, ectoplacental cone; pe, parietal endoderm; t, trophoblast; ys, visceral yolk sac.

Table 3. *The proportion and percentage of hybridization signals in various tissues of diploid hemizygous and homozygous embryos of different ages detected by* in situ *hybridization*

Tissue	Age (days)	No. of embryos scored	Total nuclei scored	Nuclei with 0 signal	Nuclei with 1 signal	Nuclei with 2 signals	% positive nuclei with 2 signals
		Diploid embryos, homozygous (5 μm sections)					
Embryo	6·5	3	123	21	46	56	54·9
	7·5	2	98	21	31	46	59·7
	8·5	3	142	25	54	63	53·8
Parietal endoderm	6·5	2	65	14	28	23	45·1
	7·5	3	193	55	67	71	51·4
	8·5	3	327	70	138	119	46·3
Ectoplacental cone	6·5	3	63	14	21	28	57·1
	7·5	2	43	12	17	14	45·2
	8·5	3	64	16	20	28	58·3
Amnion	8·5	1	78	22	31	25	44·6
		Diploid embryos, hemizygous (5 μm sections)					
Embryo	6·5	3	121	46	72	3	4·0
	7·5	3	124	49	72	3	4·0
	8·5	1	50	10	38	2	5·0
Parietal endoderm	6·5	3	91	39	51	1	1·9
	7·5	3	167	53	111	3	2·6
	8·5	1	154	57	92	5	5·1
Ectoplacental cone	6·5	3	57	27	29	1	3·3
	7·5	2	39	17	22	0	0
	8·5	1	26	11	14	1	6·7
Yolk sac (whole)	8·5	1	45	11	34	0	0
Trophoblast	7·5	1	16	6	9	1	10

displaying increasing complexity with age. By comparison, Fig. 1*d–g* (7·5, 8·5, 9·5 and 10·5 days old, respectively) illustrates typical morphology of tetraploid conceptuses. Extraembryonic membranes survived better than embryonic tissues which were much reduced and disorganized. The parietal endoderm, yolk sac and trophoblast were usually visible (Fig. 1*g*), and were the least affected structures in the tetraploid conceptuses. This agrees with the observations of Tarkowski *et al.* (1977), who also reported that the major problem during tetraploid development was the lack of mesoderm, and that mesoderm present was limited to the fetal membranes. In some cases the tetraploid conceptuses were surrounded by blood islands outside the parietal endoderm.

(iii) *Scoring hybridization signals*

In situ hybridization was carried out on all slid which had sections containing the embryo. Sectio showing labelled nuclei after *in situ* hybridizatic (indicating the presence of embryonic tissue) we noted and a section near the middle of the range w chosen for scoring (Fig. 2). By counting the number hybridization signals visible in the nuclei of differe tissues, it was possible to determine whether the tiss was hemizygous for the transgene, one spot p nucleus or homozygous (two spots). Tetraplo embryos produced from diploid hemizygous (*Tg*/ embryos should have two copies of the transgene p nucleus (*Tg*/*Tg*/−/−) (Fig. 2*a*), as the homozygo diploid (*Tg*/*Tg*) embryos do (Fig. 2*d*). The number

Fig. 2. (*a*, *b*) 'Hemizygous', *Tg*/*Tg*/−/− tetraploid mouse conceptuses, after *in situ* hybridization to detect the transgenic β-globin sequence (*Tg*) in histological sections, at (*a*) 7·5 days and (*b*) 10·5 days (dark field photograph of conceptus shown in Fig. 1*g*). Each conceptus has nuclei with two hybridization signals, including some trophoblast gia cells (small arrows). A mitotic figure indicated in (*a*) (heavy arrow) is shown in more detail in (*g*). (*c*, *d*) High-power photographs of nuclei from normal diploid embryos after *in situ* hybridization. (*c*) Nuclei with one hybridization signa in the parietal endoderm of a 7·5-day heterozygous, *Tg*/− diploid conceptus; (*d*) neurectoderm of an 8·5-day homozygous, *Tg*/*Tg* diploid conceptus with some nuclei with two hybridization signals. (*e–g*) High-power photograph of nuclei from 'hemizygous', *Tg*/*Tg*/−/− tetraploid embryos after *in situ* hybridization. (*e*) Parietal endoderm from 9·5-day tetraploid and (*f*) trophoblast giant cell from 10·5-day tetraploid, each with two signals per nucleus. (*g*) 'Hemizygous' tetraploid mitotic figure from 7·5-day conceptus, showing segregation of two pairs of labelled chromosomes (arrows indicate the two hybridization signals moving to each pole). Bar is 10 μm in (*c–g*), 50 μm in (*a*) and 100 μm in (*b*). Abbreviations as in Fig. 1.

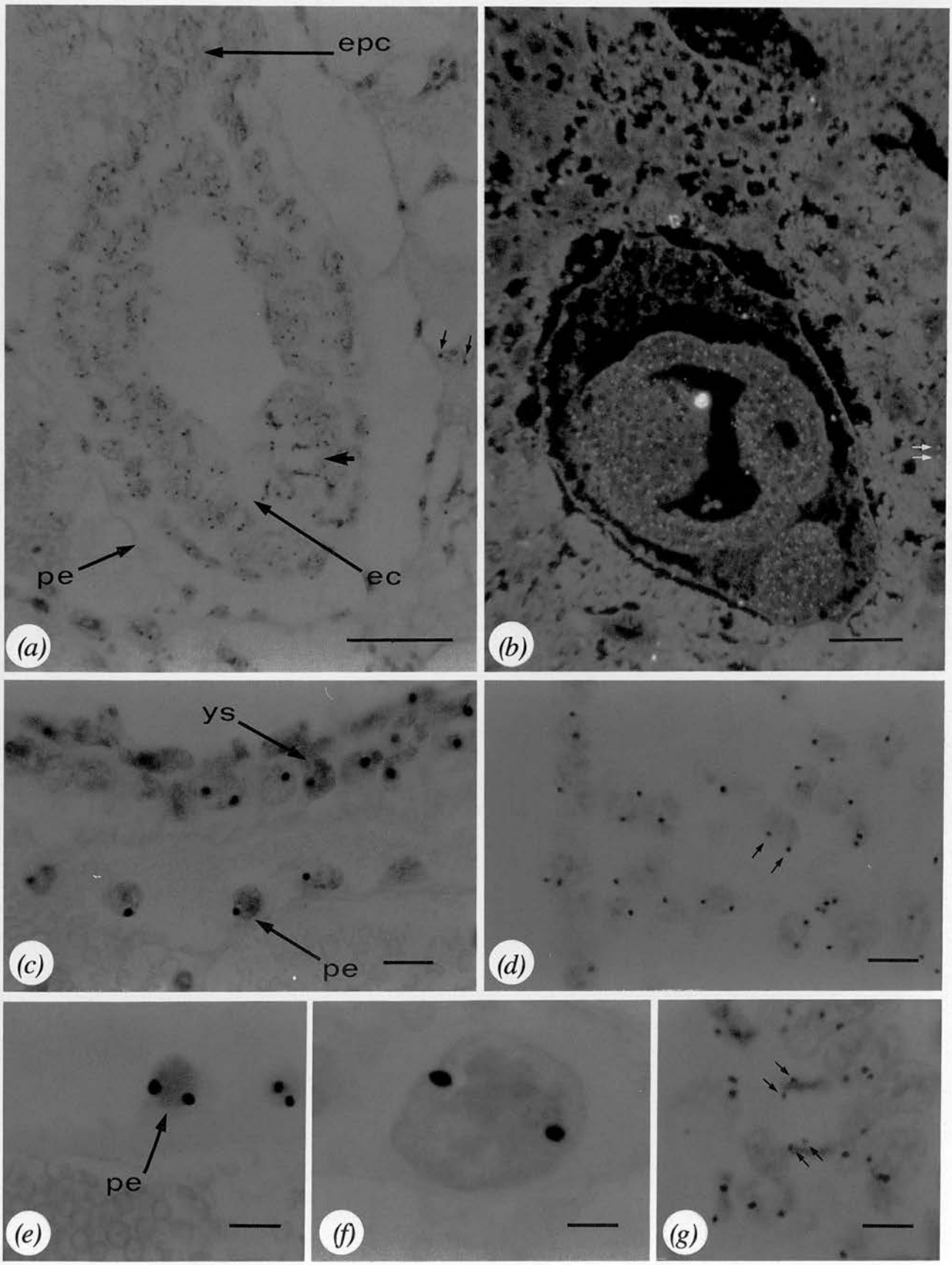

FIGURE 2.

Table 4. *Mean frequencies of nuclei with 0 or 2 hybridization signals in the embryo, parietal endoderm and ectoplacental cone*

Tissue	Expected genotype: 2n hemizygote proportion	%	2n homozygote proportion	%	4n 'hemizygote' proportion	%
Overall proportion of nuclei with no signal						
Embryo	105/295	35·6	67/363	18·5	97/410	23·7
Parietal endoderm	149/412	36·2	139/585	23·8	219/991	22·1
Ectoplacental cone	55/122	45·1	42/170	24·7	23/119	19·3
Overall proportion of positive nuclei with 2 signals						
Embryo	8/190	4·2	165/296	55·7	164/313	52·4
Parietal endoderm	9/263	3·4	213/446	47·8	451/772	58·4
Ectoplacental cone	2/67	3·0	70/128	54·7	57/96	59·4

Table 5. *Statistical significance (χ^2 test*) of the differences, in frequency of hybridization signals per nucleus, between tetraploid and both groups of diploid conceptuses*

	No signal		2 signals	
	χ^2	P	χ^2	P
2n hemizygous versus 4n 'hemizygous'				
Embryo	11·95	*$P = 0{\cdot}0005$*	119·86	*$P < 0{\cdot}0001$*
Parietal endoderm	29·76	*$P < 0{\cdot}0001$*	238·09	*$P < 0{\cdot}0001$*
Ectoplacental cone	18·25	*$P < 0{\cdot}0001$*	51·92	*$P < 0{\cdot}0001$*
2n homozygous versus 4n 'hemizygous'				
Embryo	3·12	$P = 0{\cdot}0775$	0·69	$P = 0{\cdot}4075$
Parietal endoderm	0·58	$P = 0{\cdot}4468$	12·96	*$P = 0{\cdot}0003$*
Ectoplacental cone	1·16	$P = 0{\cdot}2812$	0·49	$P = 0{\cdot}4835$

* Yates correction was applied when expected frequencies fell below 10.
Differences were considered significant if $P < 0{\cdot}05$ (shown in italics).

spots per nucleus counted in the tetraploid embryos were compared with those counted in control hemizygous and homozygous diploid embryos. A uniformly 'hemizygous' tetraploid tissue ($Tg/Tg/-/-$) would be expected to have a similar proportion of double-spot nuclei as the homozygous (Tg/Tg) controls. A mosaic tissue which was a mixture of $Tg/Tg/-/-$ and $Tg/-$ cells would have a lower percentage of nuclei with 2 spots.

The tissues scored were: embryo, parietal endoderm, ectoplacental cone and trophoblast (Fig. 1 *a–g*). All four of these structures were easy to distinguish in early stage conceptuses. Embryonic and extra-embryonic tissues were included in the scoring system to ensure that if 4n/2n mosaicism was confined to one developmental lineage, it would still be detected. Apart from the parietal endoderm where the whole structure was scored, areas of tissue to be counted were delineated under a Leitz Diaplan microscope using an eye-piece with 1 mm grid. Using a ×25 objective and ×12·5 (20 mm) eye-piece, a single square, (corresponding to 50 × 50 μm on the slide) was selected and each of the nuclei contained in that area was scored as: 0 signal; 1 signal; 2 signals or unscoreable. One area each of trophoblast tissue and ectoplacental cone tissue was scored for each embryo, and two areas of embryonic tissue.

The mean and the standard error for the percentage of each of the categories in the scoring system: 0 signal; 1 signal; 2 signals or unscoreable, were calculated for each age of embryo, for both the tetraploid and diploid series. The number of embryos and the total number of nuclei scored for each age and tissue can be seen in Tables 2 and 3 (tetraploid and diploid respectively). The proportion of nuclei with no signal gives an indication of the percentage of false negatives which occurred due to failure of the *in situ* technique. Also calculated was the proportion of positive nuclei with two signals. The total proportion of nuclei with no signal and positive nuclei with two signals for each tissue type, disregarding age, is shown in Table 4. The results were tested statistically using a χ^2 test (Table 5). The data for the 'hemizygous' tetraploid embryos was tested statistically against both the hemizygous and homozygous diploid values. Tables 4 and 5 show that the 'hemizygous' tetraploid

Table 6. *Statistical significance (χ^2 test*) of the differences, in frequency of hybridization signals per nucleus, between tissues in tetraploid conceptuses*

	Proportion of nuclei with no signal		Proportion of nuclei with 2 signals	
Comparison	χ^2	P	χ^2	P
Embryo versus parietal endoderm	0·40	0·5251	3·29	0·0697
Embryo versus ectoplacental cone	0·99	0·3206	1·44	0·2300
Parietal endoderm versus ectoplacental cone	0·48	0·4891	0·03	0·8578

* Yates correction was applied when expected frequencies fell below 10.
No differences were considered statistically significant (none with $P < 0{\cdot}05$).

group had significantly fewer nuclei with no signal and significantly more nuclei with two signals than the hemizygous diploid group. Comparison of 'hemizygous' tetraploid and homozygous diploid groups revealed no significant difference for the embryo or ectoplacental cone. Although a significant difference in the frequency of nuclei with two signals was detected in the parietal endoderm this does not provide evidence for mosaicism in the 'hemizygous' tetraploid group because the frequency of nuclei with two signals was higher in the 4n than the homozygous diploid parietal endoderms. χ^2 tests were also carried out between the different tissues scored in tetraploid embryos (Table 6). There was no significant difference between the different tissue types tested. This supports the conclusion that the discrepancy in the parietal endoderm χ^2 values is not biologically significant.

4. Discussion

The development of the tetraploid embryos generated by this particular combination of strains was poorer than that previously reported by Kaufman & Webb (1990). Viable embryos have been recorded as late as 17·5 (Snow, 1975) and 16 days of gestation (equivalent to normal embryos of about 14–14·5 days p.c., Kaufman, 1991 *a*). There are two possible explanations for this observation; either the development of tetraploid embryos varies according to the strain combination (genetic background) or that the presence of the transgene is somehow interacting to hamper development in the tetraploid embryo in a way not observed in diploids generated from the same strain. The former explanation would seem more plausible, since strain dependent effects on development have been reported in several different instances for both tetraploidy (Tarkowski *et al.* 1977; Dyban & Baranov; 1987; Webb *et al.* 1992) and triploidy (Wroblewska, 1971; Niemierko, 1981). In the series of tetraploid mouse embryos described by Snow (1975), the embryonic ectoderm was commonly reduced and disorganized, the cells being loosely arranged and fewer in number than diploids of the same age. Tarkowski *et al.* (1977) noted that growth of tetraploid conceptuses was generally slower than in normal diploid embryos. By 8·5 days of gestation the embryonic portion of the conceptus appeared underdeveloped with either an absence of, or vestigial embryonic structures. The type of morphological changes observed in tetraploid conceptuses have also been seen in embryos with other chromosome abnormalities (Dyban & Baranov, 1987). For instance, the triploidy syndrome described by Wroblewska (1971) involved a general retardation of growth, but was also strain dependent, since in appropriate strain combinations, limb bud stage diandric and digynic triploid embryos are observed (Kaufman, 1991 *b*), though more advanced stages of development have so far not been reported. By around 8–9 days of gestation the embryonic region of the triploidy syndrome conceptus was small in comparison to the extraembryonic region. The primary germ layers were less differentiated, with fewer layers and rounded cells. These observations closely resemble morphology seen in tetraploid embryos, although the tetraploid phenotype manifests earlier and often results in no more than a trophoblastic vesicle (Graham, 1971; Snow 1976). Tetraploid embryos have been recovered at between 10 days of gestation (Tarkowski, 1977) which resembles more closely the conceptuses collected in this study, and 17·5 days of gestation reported by Snow (1975). However, recent attempts to repeat the success achieved by Snow, have resulted in embryos no older than 16 days of gestation (Kaufman, 1991 *a*). There may be two explanations for these differences. Firstly, that the differences in development are due to the strain combinations used, or that Snow's tetraploids were perhaps 4n/2n mosaics and that the cytogenetic analysis failed to identify them as such. Even a small proportion of diploid cells in a mosaic embryo may have been sufficient to partially rescue the embryo and allow development to proceed further than in a solely tetraploid embryo.

A low incidence of nuclei with two positive signals was observed in all tissues of the hemizygous diploid ($Tg/-$) conceptuses (Table 3). There are two possibl

explanations for this observation. Firstly, that the cells are genuinely tetraploid, as observed, for example in the trophoblast. Alternatively, the apparent polyploid cells observed may be technical artefacts, due to overlapping cells, despite the fact that the sections were cut at 5 μm to compensate for the smaller nuclear diameter of the diploid cells. The latter seems less likely since closely packed cells or cells which did not have obvious nuclear borders were counted as unscoreable.

The incidence of 2 signals per nucleus in homozygous diploid (and 'hemizygous' tetraploid) conceptuses is only $\sim 50\%$, when, theoretically, the expected value is 100%. This is most likely to be an artefact attributable to the use of histological sections, because some target sequences will be excluded from part of the sectioned nuclei particularly in thin sections. The ability to detect mosaicism is reduced by the failure to detect both hybridization signals in about 50% of the nuclei expected to have two copies of the transgene. Nevertheless it should have been possible to recognize the presence of diploid cells in the tetraploid embryos, if they comprised at least 15% of the total nuclei scored, as explained below.

If the proportion of all nuclei scored that had one hybridization signal in the embryonic region of the 4n group was 177/410 (43·2%), instead of 149/410 (36·3%; Table 2), then this would have been significantly higher than the corresponding proportion for the homozygous diploid embryos; 131/363; 36·1% ($\chi^2 = 4{\cdot}03$; $P = 0{\cdot}045$). This higher proportion of nuclei with one signal could have been accounted for by 11·1% of diploid nuclei in the 'tetraploid' embryos; as calculated by:

$$\left[1-\left(\frac{0+2 \text{ spots in 4n}}{\text{All nuclei in 4n}}\Big/\frac{0+2 \text{ spots in 2n homozygotes}}{\text{All nuclei in 2n homozygotes}}\right)\right]\times 100.$$

This formula is based on that used to calculate the number of induced mutations (Lyon, 1970) and, when used for the above example, resolves as

$$\left[1-\left(\frac{233}{410}\Big/\frac{232}{363}\right)\right]\times 100 = 11{\cdot}1\%.$$

Therefore, on this basis, it would have been possible to detect the presence of diploid cells in the tetraploid embryos if they composed at least 11·1% of the total nuclei scored. A similar calculation, ignoring the nuclei with no hybridization signals, predicted that 2n/4n mosaicism would be detected if at least 14·6% of the nuclei were diploid. Thus, diploid cells in the tetraploid embryos should have been detected, by a statistically significant increase in the proportion of nuclei with only one signal, if they comprised at least 15% of the total nuclei scored.

The frequency of nuclei with no hybridization signal was higher in the hemizygous diploid series. This was expected since there is a greater chance of failing to include the target sequence in a section when there is only one target per nucleus, compared to two sequences in both the homozygous diploid and 'hemizygous' tetraploid series. The frequency of positive nuclei with two signals in 'hemizygous' tetraploid ($Tg/Tg/-/-$) conceptuses was not significantly lower than that observed in the homozygous diploid (Tg/Tg) conceptuses. This implies that the tissues in the tetraploid conceptuses are uniformly 4n, and consequently that there is no evidence that the electrofusion technique results in 4n/2n mosaicism.

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ıantitative analysis of mid-gestation mouse aggregation chimaeras: n-random composition of the placenta

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ract. Mouse chimaeras were produced by aggregat-ight-cell embryos from two different F_2 matings, ab-ated to AF_2 and BF_2 respectively: (C57BL/AKR-*Gpi-1s*^{a}, *c*/Ws female × BALB/c male)F_2 and BL/Ws female × CBA/Ca male)F_2. Quantitative rophoresis of glucose phosphate isomerase (GPI-1) ısed to estimate the proportions of the two cell pop-ons in different tissues of the $12^1/_2$ day chimaeric ɛptuses, with the % GPI-1A indicating the percent-f cells derived from the AF_2 embryos. The % GPI-ɣas found to be highly positively correlated within rimitive ectoderm lineage (between the fetus, am-and yolk sac mesoderm) and within the primitive derm lineage (between the yolk sac endoderm and arietal endoderm) but no correlation (either positive gative) was seen between the two lineages. This con-the results of a previous study of chimaeras made ɛen partially congenic strains and suggests the origi-onclusions have general validity. The % GPI-1A in acenta was corrected for the expected contribution ternal GPI-1, based on control experiments involv-ansfer of homozygous *Gpi-1s*^{b}/*Gpi-1s*^{b} embryos to eri of *Gpi-1s*^{a}/*Gpi-1s*^{a} pseudopregnant females. The cted % GPI-1A in the placenta was positively corre-with that in each of the three primitive ectoderm ıtives. This suggests either (1) exchange of cells be-the polar trophectoderm and the underlying part inner cell mass that forms the primitive ectoderm cells are incompletely mixed in the chimaeric blas-and patches of AF_2 and BF_2 cells straddle the lary between the polar trophectoderm and the un-ng primitive ectoderm. The second explanation ıot imply the existence of shared developmental es between trophectoderm and primitive ectoderm ı-chimaeric embryos. Unlike that of any other tis-ıe distribution of placental GPI-1A was U-shaped; 28 placenta samples the proportion of the minor ɔnent was 10% or less. This suggests that the pla-trophoblast is derived from a small number of co-herenct clones of polar trophectoderm cells (either a small number of polar trophectoderm cells or a larger number if the two cell populations are not finely intermingled). Thus, although as a population the placentas of chimaeric conceptuses are balanced with respect to the % GPI-1A (mean close to 50%), individually most placentas are extremely unbalanced in their chimaeric composition (< 10% or > 90% GPI-1A). This non-random composition of the chimaeric placentas is in contrast to the widely held assumption that the distribution of cells in chimaeric conceptuses is normally random.

Key words: Chimaera – Mosaic – Mouse – Fetus – Placenta

ondence to: J.D. West

Introduction

Mouse embryos with various types of developmental defects have been incorporated into aggregation chimaeras or injection chimaeras in order to investigate their developmental potential. Those incorporated into aggregation chimaeras include androgenetic, parthenogenetic and gynogenetic embryos (Nagy et al. 1987, 1989; Surani et al. 1987, 1988; Thomson and Solter 1988, 1989; Fundele et al. 1989, 1990, 1991), embryos with various chromosome anomalies (Epstein et al. 1982, 1984; Cox et al. 1984; Fundele 1985) as well as embryos with lethal single gene defects, such as various t-haplotypes (Mintz 1964; Bennett 1978) and lethal yellow (Barsh et al. 1990). One advantage of using aggregation chimaeras is that both cell populations may contribute to the fetus and all of the extraembryonic tissues. Although, in many cases investigations have been limited to fetal or adult tissues, in some cases the placenta (Cox et al. 1984; Epstein et al. 1984) and other extraembryonic tissues (Nagy et al. 1987; Surani et al. 1987, 1988; Thomson and Solter 1988, 1989) have also been considered. This has provided additional valuable information about the developmental potential of the abnormal cell type, particularly in

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he case of cells from androgenetic, parthenogenetic and gynogenetic embryos.

A series of elegant experiments with injection chimaeras by Gardner and his colleagues helped to establish he developmental fates of the three primary developmental lineages: the primitive ectoderm or epiblast, the primitive endoderm or hypoblast and the trophectoderm eg. Gardner and Papaioannou 1975; Gardner 1978). n a subsequent study of $12^1/_2$ day chimaeric conceptuses made by aggregating two normal embryos, that differed at the *Pgk-1* locus, the proportions of cells derived from each embryo were analysed in the fetus and various extraembryonic tissues (West et al. 1984). This study revealed positive correlations between three tissues derived from the primitive ectoderm (fetus, amnion and yolk sac mesoderm) and between the two primitive endoderm derivatives (yolk sac endoderm and parietal endoderm). There was, however, no correlation between the derivatives of primitive ectoderm and the primitive endoderm. Thus, the proportions of the two cell populations were positively correlated within each of the two developmental lineages studied but no correlations were seen between developmental lineages.

These quantitative relationships established for control aggregation chimaeras provide a valuable baseline for evaluating the developmental potential of cells, with various types of developmental defects, in the fetus and extraembryonic tissues. For example, if comparison of control and experimental chimaeras (involving a genetically abnormal cell population) revealed a difference in correlation between tissues or a change in slope of a regression line, this might reveal which tissues were most affected by the genetic defect.

In the previous study by West et al. (1984), chimaeras were made by aggregating embryos of the inbred C3H/HeH strain with embryos of the partially congenic strain C3H/HeHa-*Pkg-1*^{a} and the proportions of cells derived from each embryo were estimated by quantitative electrophoresis of phosphoglycerate kinase-1 (PKG-1; EC 2.7.2.3). In the present study we have made chimaeras by aggregating two types of F_2 embryos that differ at the *Gpi-1s* locus and the proportions of cells derived from each embryo were estimated by quantitative electrophoresis of glucose phosphate isomerase (GPI-1; EC 5.3.1.9).

The aims of this study were twofold. First, we wanted to test whether the statistical correlations revealed by the earlier study could be confirmed using chimaeras of a different strain combination that were analysed by a different method. (The level of genetic disparity, both within and between groups of F_2 embryos used in the present study, was higher than that in the earlier study, where partially congenic strains were used.) Second, we wanted to extend the analysis to include tissues derived from the third primary developmental lineage, the trophectoderm.

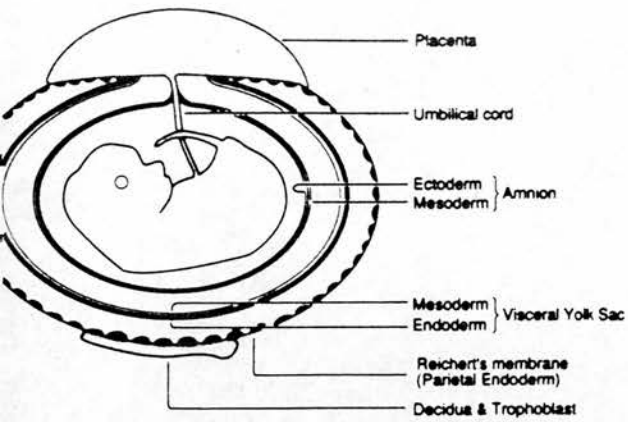
Materials and methods

Production of chimaeras. Two groups of F_1 mice were produced. The F_1 stock (abbreviated to AF_1) was produced by crossing inbred, partially congenic C57BL/Ola.AKR-*Gpi-1s*^{a}.*c*/Ws strain females (name abbreviated to BC) with inbred BALB/c males. The other F_1 (abbreviated to BF_1) was produced by crossing inbred C57BL/Ws strain females with inbred CBA/Ca males. The AF_1 animals were homozygous for albino (*c/c*) and the *Gpi-1s*^{a} allele of the glucose phosphate isomerase locus; the BF_1 animals were homozygous *C/C* (pigmented) and *Gpi-1s*^{b}/*Gpi-1s*^{b}. CBA/Ca males were obtained from the Institute of Cell, Animal and Population Biology, University of Edinburgh. BALB/c mice were purchased from the Department of Medical Microbiology, University of Edinburgh; all other animals were bred and maintained, under conventional conditions in the Centre for Reproductive Biology.

AF_1 and BF_1 females were superovulated by injecting 5 IU pregnant mares' serum gonadotrophin (PMSG) at approximately 12 noon followed 48 hours later by 5 IU human chorionic gonadotrophin (hCG). Females were housed with males from the same F_1 strain and mating verified the following morning by the presence of a vaginal plug; the day of the vaginal plug was designated $^1/_2$ day *post coitum* (*p.c.*). Embryos produced in this way were $(AF_1 \times AF_1)F_2$ and $(BF_1 \times BF_1)F_2$; abbreviated to AF_2 and BF_2 respectively. Unlike their F_1 parents, the F_2 embryos would be genetically heterogeneous; AF_2 embryos would be segregating at loci that differed between the inbred strains BC and BALB/c and BF_2 embryos would be segregating at loci that differed between the inbred strains C57BL and CBA. On the day that the vaginal plugs were found, another group of AF_1 females were examined and those in oestrous were mated to vasectomised males to provide pseudopregnant females.

Preimplantation embryos were flushed from the reproductive tract of pregnant AF_1 and BF_1 females at $2^1/_2$ days *p.c.* (usually between 8.30 am and 11.00 am) with HEPES-buffered M2 handling medium (Quinn et al. 1982). Most were at the 8-cell stage; the zonae pellucidae were removed in acidic Tyrodes solution, pH 2.5 (Nicolson et al. 1975) and the denuded embryos were washed in M2 medium. Aggregation chimaeras were produced by standard procedures involving aggregation of pairs of zona-free 8-cell stage embryos (Tarkowski 1961; Mintz 1962; McLaren 1976). Embryos were transferred in pairs (one AF_2 and one BF_2 embryo) to drops of culture media under paraffin oil (Boots) in bacteriological grade petri dishes. Firstly, each pair was pushed together in a drop of M2 + PHA [1 part phytohaemagglutinin (M form, GIBCO 670-0576) plus 19 parts M2 medium] to aid adhesion (Mintz et al. 1973; Pratt 1987), incubated for 2 min at 37° C in 5% CO_2 in air, then rinsed in a drop of M2. The aggregated embryos were then rinsed in a drop of M16 culture medium (Whittingham 1971) and transferred to a second drop of M16 medium under paraffin oil dish, in a dish that had been equilibrated overnight at 37° C in 5% CO_2 in air. Any pairs of embryos that had drifted apart were pushed together again and the aggregated pairs were cultured overnight. (Some developmental asynchrony is likely between the aggregated embryos because they were aggregated soon after collection, without checking the time of their previous cleavage division.) The following day the aggregated embryos were rinsed in M2 handling medium and surgically transferred to the uterus of an AF_1 pseudopregnant female, using a fine glass pipette (McLaren and Michie 1956). Pseudopregnant females were anaesthetised with 0.25 ml per 30 g body weight of a 1:1 v/v mixture of Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone; Janssen pharmaceuticals) and Hypnovel (2 mg/ml midazlam hydrochloride; Roche). Pregnancies were timed according to the pseudopregnant female.

Analysis of chimaeras. Females were sacrificed at $12^1/_2$ days gestation and the conceptuses were dissected as described by Papaioannou and West (1981) to provide fetus, amnion, visceral yolk sac, Reichert's membrane (parietal endoderm), umbilical cord and placenta (see Fig. 1 for diagram of conceptus). The mixture of decidua and trophoblast tissue was carefully removed from Reichert's membrane with watchmaker's forceps and the endoderm and mesoderm layers of the yolk sac were separated with watchmaker's forceps after incubating the whole yolk sac in trypsin pancreatin at 4° C



1. Diagram of $12^1/_2$ day mouse conceptus (re-drawn after Pa-annou and West 1981). The different tissues are shaded accord-to their developmental lineage. The fetus, amnion and yolk nesoderm (*unshaded*) are derived from the epiblast or primitive derm lineage; the visceral yolk sac endoderm and parietal en-rm of Reichert's membrane (*shaded black*) are derived from rimitive endoderm lineage; the placenta and band of tissue he outer surface of Reichert's membrane (*shaded grey*) are ly a mixture of trophoblast (*trophectoderm lineage*) and mater-lecidua tissue, although the placenta has minor contributions other lineages

$^1/_2$–3 h (Levak-Svajger et al. 1969) and rinsing in M2 handling um. The weights of the total conceptus, the placenta and the were recorded, along with the crown/rump length and mor-gical index based on hind limb development (McLaren and r 1990; Palmer and Burgoyne 1991).

he fetus and placenta were stored at −20° C, in 100 µl of glycerol in water, in 1.5 ml microtubes and all other tissues stored in 10 µl of 50% glycerol in microtest plates. Samples lysed by three cycles of freeze/thawing with mechanical dis-n of the fetal and placental tissues. Electrophoresis, staining ucose phosphate isomerase (GPI-1) activity and densitometry a Helena Process-24 gel scanner) were carried out as previous-scribed (West et al. 1986). The proportions of the two cell -ations in the chimaeric tissues were estimated from the pro-ns of GPI-1A and GPI-1B allozymes. The raw data (as % A allozyme given to one decimal percentage point) was used atistical analysis and plotting the figures but they were ed to the nearest integer for presentation in Table 2. Non-netric statistical tests were performed on an Apple Macintosh computer using the "MultiStat" statistical package (Biosoft, Cambridge).

The accuracy of the quantification of the two GPI-1 allozymes by cellulose acetate electrophoresis and scanning densitometry was tested using samples with known proportions of GPI-1A and GPI-1B. This was achieved by mixing kidney homogenates, from homozygous *Gpi-1s*^{a}/*Gpi-1s*^{a} mice with those from homozygous *Gpi-1s*^{b}/*Gpi-1s*^{b} mice, in different proportions, coding the samples and estimating the percentage GPI-1A by quantitative electrophoresis. As reported previously (eg. West and Green 1983) there was a slight tendency to overestimate the minor component but overall the results showed a very good concordance between the observed and expected percent GPI-1A.

Analysis of control conceptuses. Homozygous, *Gpi-1s*^{a}/*Gpi-1s*^{a} (AF^1) pseudopregnant females were used as recipients for the chimaeric embryos, so any maternal contamination would increase the proportion of GPI-1A in the tissue. In order to assess the extent of the maternal contribution to the different tissues analysed a control series of embryo transfer was undertaken. Homozygous, *Gpi-1s*^{b}/*Gpi-1s*^{b} (BF_2) preimplantation embryos were flushed from the reproductive tract of BF_1 females at $3^1/_2$ days and surgically transferred to the uteri of pseudopregnant AF_1 females. Females were sacrificed at $12^1/_{12}$ days gestation and the conceptuses were dissected and analysed by GPI-1 electrophoresis as described above for the chimaeric group.

In situ hybridisation of placenta. The maternal and "fetal" parts of the placentas of male conceptuses were distinguished by in situ hybridisation with the Y chromosome-specific probe, pY353/B (kindly provided by Dr. Colin Bishop, Institut Pasteur, Paris, France). The probe was labelled with digoxygenin and detected, after in situ hybridisation, by alkaline phosphatase immunocytochemistry as described by Keighren and West (1993).

Results

Transfer control conceptuses

Table 1 shows the percentage of maternally-derived GPI-1A activity in various parts of 34 conceptuses derived from the transfer of homozygous, *Gpi-1s*^{b}/*Gpi-1s*^{b} preimplantation embryos transferred to homozygous *Gpi-1s*^{a}/*Gpi-1s*^{a} females. This analysis revealed that while the fetus, amnion, yolk sac mesoderm, umbilical cord and yolk sac endoderm showed no contamination, the parie-

1. Proportion of GPI-1A (maternal-ived enzyme) in various tissues of 34 -day conceptuses produced by trans-; homozygous *Gpi-1s*^{b}/*Gpi-1s*^{b} preim-tion embryos into homozygous *Gpi-i-1s*^{a} pseudopregnant recipient fe-

Tissue	Proportion of conceptuses with GPI-1A	% GPI-1A (maternal contamination)		
		Mean ±SEM	Coefficient of variation (%)	Range
Fetus	0/34	0		
Amnion	0/34	0		
Yolk sac mesoderm	0/34	0		
Umbilical cord	0/34	0		
Yolk sac endoderm	0/34	0		
Parietal endoderm	14/33	3.3 ± 2.6	140.1	0–19
Decidua and trophoblast	34/34	74.6 ± 1.8	20.8	44–100
Placenta	34/34	44.3 ± 1.4	20.4	12–64
Placenta[a]	33/33	45.3 ± 1.0	15.5	32–64

[a] Second row of placenta data excludes one placenta with only 11.5% GPI-1A. This placenta was abnormally light and it is likely that most of the outer maternal layer was lost during dissection

Fig. 2. A 5 μm histological section of the placenta and part of the embryo of a non-chimaeric, $11^1/_2$ day, male, mouse conceptus after in situ hybridisation to Y chromosome probe, pY353/B labelled with digoxygenin. The placenta appears as a crescent-shaped structure that fills most of the photograph and the presence of the Y chromosome is detected by dark (blue) staining of alkaline phosphatase (see Materials and methods). Since, maternal cells have no Y chromosome, the maternal part of the placenta (m) is clearly identified as the pale outer region that fails to stain for alkaline phosphatase. *Bar* is 1 mm

tal endoderm, decidua/trophoblast and the placenta each had a significant proportion of GPI-1A. Although none of the umbilical cord samples had any maternal contamination, this tissue was not analysed in the chimaera series (below) because the blood content was variable. Fourteen of 33 parietal endoderm samples were contaminated with maternally-derived GPI-1A but since the overall mean was only 3.3% this was not taken into account when analysing the parietal endoderm samples of chimaeric conceptuses. The mixture of decidua and trophoblast tissue dissected from Reichert's membrane was predominantly GPI-1A and 5/34 samples were 100% GPI-1A (presumable entirely decidua). For this reason it was not feasible to correct for the proportion of maternal tissue in the decidua/trophoblast of chimaeric conceptuses, so this sample was not analysed in the chimaeric series.

The placenta, on the other hand, had a high but less variable proportion of maternal GPI-1A. This presumably reflects the contribution of the outer maternal layer of the placenta that is derived from the uterine decidua (Fig. 2) plus maternal blood. One placenta had a much lower proportion of GPI-1A (11.5%) than all of the others (mean 44.3%; range 31.5–64.4%). This placenta was also the lightest (0.06 g compared to a mean of 0.09 g) and had the highest fetus/placenta weight ratio (2.00 compared to a mean of 1.29 and a range of 0.80 to 1.67 for the others). It seems likely that the outer, maternal part of the placenta was lost during the removal of this conceptus from the uterus, thereby accounting for its low weight and low proportion of maternally-derived GPI-1A. Ignoring this placenta, the mean proportion of GPI-1A in the remaining 33 placentas was approximately 45% (Table 1) and this figure was used to correct the proportion of GPI-1A present in the chimaeric placentas (below).

Chimaeric conceptuses

A series of 39 conceptuses was produced (Table 2), including five that were non-chimaeric in all the tissues studied, one set of twins within a single amnion and a pair of conceptuses with fused placentas. The two fused placentas were separated at dissection but, in case the separation was incomplete, these conceptuses were excluded from the statistical analysis. The results from the remaining 28 separate chimaeric conceptuses were analysed statistically.

Electrophoresis of GPI-1A from chimaeric samples is illustrated in Fig. 3. The observed placental % GPI-1A

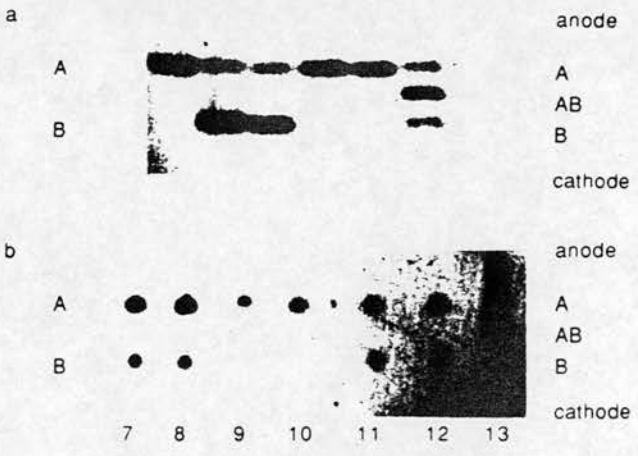


Fig. 3a, b. GPI-1 electrophoresis plates. **a** Samples loaded with a 0.25 μl applicator: lane 1, fetus XE-1; 2, fetus XE-2; 3, fetus XE-5; 4, fetus XE-6; 5, fetus XE-7; 6, control blood sample from a *Gpi-1s*^{a}/*Gpi-1s*^{b} heterozygote producing GPI-1A, GPI-1AB and GPI-1B allozymes. **b** Samples from conceptus XE-38, loaded from a fine Pasteur pipette: lanes 7 & 8, yolk sac mesoderm; 9 & 10, parietal endoderm; 11 & 12, amnion; lane 13, control blood sample from a *Gpi-1s*^{a}/*Gpi-1s*^{b} heterozygote. Allozymes (A, AB and B) are inidcated at the side. Migration was towards the cathode

GPI-1A in tissues of $12^1/_2$ day chimaeric conceptuses, ranked by % GPI-1A in the fetus

ra	% GPI-1A							
	Primitive ectoderm lineage			Primitive endoderm		Trophectoderm lineage		
	Fetus	Amnion	Yolk Sac mesoderm	Yolk Sac endoderm	Parietal endoderm	Placenta	Placenta corr-1*	Placenta corr-2*
	22	28	24	56	71	41	−7	0
	26	43	22	43	93	45	0	0
	28	32	35	31	46	68	42	42
	30	25	29	17	36	44	−2	0
	41	43	45	43	37	50	9	9
	43	50	53	16	65	50	9	9
	47	47	50	68	53	58	24	24
	51	48	45	43	61	22	−42	0
	55	50	59	44	33	65	36	36
	63	61	49	21	58	48	5	5
	64	80	65	48	48	91	83	83
	68	77	78	59	42	56	19	19
	69	67	58	15	35	45	0	0
	71	85	64	54	79	81	66	66
	72	55	64	26	51	46	1	1
	72	78	72	37	39	99	98	98
	72	48	59	87	89	95	91	91
	73	81	72	23	85	98	96	96
	85	84	76	99	100	98	96	96
	85	85	94	47	100	54	16	16
	87	68	87	62	100	89	79	79
	88	81	71	52	46	90	82	82
	90	91	93	43	93	94	90	90
	91	88	94	92	100	100	100	100
	95	88	96	45	49	94	89	89
	100	100	100	52	77	100	100	100
	100	100	100	35	34	100	100	100
	100	100	100	41	16	46	2	2
	67.41	67.24	66.18	46.36	61.96	70.18	45.77	47.61
	4.51	4.30	4.48	4.07	4.78	4.66	8.46	7.93
	23.89	22.74	23.72	21.52	25.31	24.63	44.79	41.97
	28	28	28	28	28	28	28	28
.	35.44	33.82	35.84	46.42	40.85	35.11	97.85	88.15
ıses with fused placentas								
	67	56	67	78	91	87	77	77
	50	53	44	43	83	35	−18	0
thin a single amnion								
	89	84	82	31	18	44	−3	0
	98							
ıaeric conceptuses								
	0	0	0	0	0	44	−2	0
	100	100	100	100	100	100	100	100
	100	100	100	100	100	100	100	100
	100	100	100	100	100	100	100	100
	100	100	100	100	100	100	100	100

cental value was corrected to allow for 45% of maternal GPI-1 in the placenta. Placenta corr-1: % GPI-1A = [(observed A–45) × 100]/(100–45). In placenta corr-2, the negative values are treated as 0

rected to allow for an average of 45% placental ctivity that was of maternal origin. Since 45% erage figure, this results in an over-correction cases and resulted in three negative values he 28 conceptuses included for statistical analy- negative values were used to calculate the corre- lation coefficients but were counted as zero for plotting the figures. Conceptus XE-26 had a very low corrected estimate for the % GPI-1A in the placenta (−41.8%); the uncorrected estimate was 22.0% GPI-1A. This placenta was also lighter than most and was ranked joint second in terms of fetus placenta weight ratio (2.00 com-

Table 3. Probability (P) values from Mann-Whitney U-tests, comparing % GPI-1A in different tissues of 28 chimaeric conceptuses. Tissues are grouped according to their origin from primitive ectoderm, primitive endoderm or trophectoderm developmental lineages

	Primitive ectoderm		Primitive endoderm		Trophectoderm Placenta (corrected)
	Amnion	Yolk Sac mesoderm	Yolk Sac endoderm	Parietal endoderm	
Fetus	0.8722	0.7304	*<0.0001*	0.2752	*0.0299*
Amnion		0.8632	*<0.0001*	0.2701	*0.0336*
Yolk Sac mesoderm			*<0.0001*	0.3066	*0.0376*
Yolk Sac endoderm				*0.0023*	0.6133
Parietal endoderm					*0.0482*

Differences in mean % GPI-1A between tissues were considered to be statistically significant when $P < 0.05$ (shown in italics)

pared to a mean of 1.25 and a range of 0.67 to 2.50). t seems probable that, like one of the control conceptuses (above), the outer, maternal part of the placenta was lost during the removal of this conceptus from the uterus, resulting in a low weight and low proportion of maternally-derived GPI-1A.

The distributions of the % GPI-1A in the 28 chimaeric conceptuses were plotted as histograms for each of the six tissues analysed (Fig. 4). These histograms, and the mean values in Table 2, show that for the fetus, amnion, yolk sac mesoderm and the parietal endoderm the distributions are slightly skewed in favour of a higher percentage of GPI-1A (AF_2 cells) but for yolk sac endoderm the distribution shows more equal proportions of GPI-1A and GPI-1B. The distribution of % GPI-1A for placental samples (corrected values) is U-shaped, with few samples showing a balanced proportion of GPI-1A and GPI-1B. Consequently, the standard deviation for the corrected placentas is higher than for other tissues (Table 2). Statistical analysis with the Kruskal-Wallis test showed that there was significant heterogeneity in the mean % GPI-1A among tissues (Kruskal-Wallis statistic = 14.742; $P = 0.012$). Further analysis with the Mann-Whitney U-test on data from pairs of tissues (Table 3) confirmed the visual impressions from Fig. 4 and showed that the mean GPI-1A was similar in the fetus, amnion, yolk sac mesoderm and parietal endoderm but differed from those in the yolk sac endoderm and the placenta. The statistical significance was higher for the difference between the yolk sac endoderm and the other four tissues than between the placenta and these other tissues. The Mann-Whitney test showed no significant difference, in the mean % GPI-1A, between the yolk sac endoderm and the placenta but Fig. 4 shows that, while the means are similar, the two distributions are completely different. This difference was found to be statistically significant by a χ^2 test with three degrees of freedom ($\chi^2 = 18.86$; $P < 0.001$). [In order to ensure expected values of greater than 5, the data for yolk sac endoderm and placenta samples (data as in Table 2 but to one decimal place) were ranked together (56 samples) and then divided into quartiles according to their rank order (14 samples per group). A χ^2 test was performed to compare the distribution of the 28 yolk sac endoderm ans 28 placenta samples among the four quartiles (3, 10, 12, 3 versus 11, 4, 2, 11).]

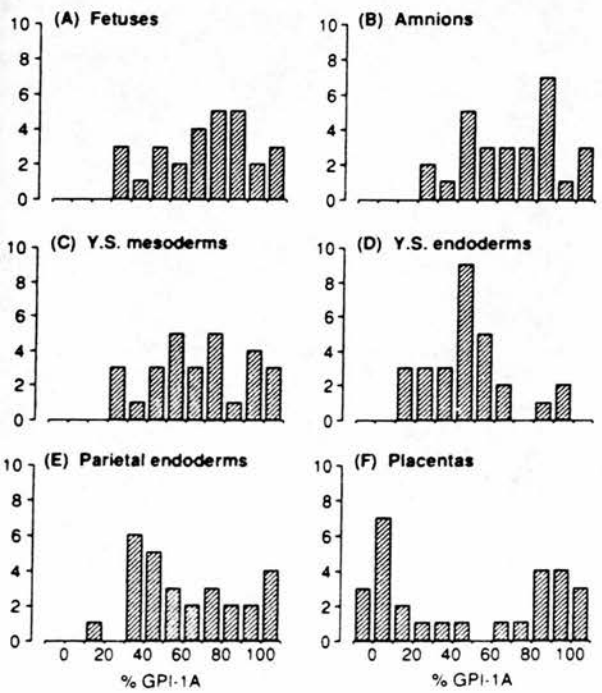


Fig. 4. Distribution of % GPI-1A in the six tissues analysed from chimaeric conceptuses. Tissues with either 0% or 100% GPI-1A are shown separately at either end of the distribution

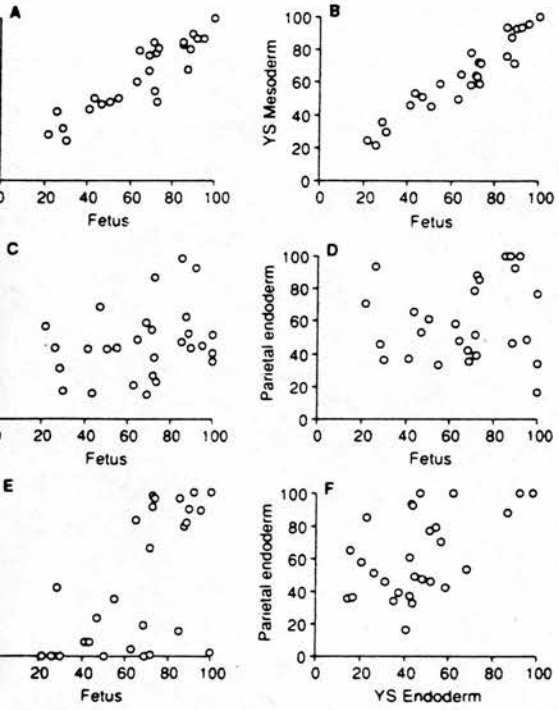
The relationships in the % GPI-1A between pairs of tissues are shown in Fig. 5 and statistical correlations are shown in Table 4. The statistical analyses show that the % GPI-1A was significantly positively correlated within the primitive ectoderm derivatives (fetus, amnion and yolk sac mesoderm) and within the primitive endoderm derivatives (yolk sac endoderm and parietal endoderm) but not between the tissues derived from these two different developmental lineages. The % GPI-1A in the placenta (corrected for maternal GPI-1A) was positively correlated with the three primitive ectoderm tissues and also reached borderline significance for a positive correlation with the yolk sac endoderm but not with the parietal endoderm.

The numerical data in Table 2 reveals information that is not shown in the figures or by the statistical analyses. In some chimaeric conceptuses, one cell population was excluded from one or more tissues. This occurred in 3/28 fetuses and in each of these conceptuses (XE-6, XE-17 and XE-21) the same cell population

4. Spearman rank correlation coeffi-
, r (with P values below) for % GPI-
1 different tissues of chimaeric con-
ises. Tissues are grouped according to
origin from primitive ectoderm,
itive endoderm or trophectoderm de-
mental lineages

	Primitive ectoderm		Primitive endoderm		Trophectoderm Placenta (corrected)
	Amnion	Yolk Sac mesoderm	Yolk Sac endoderm	Parietal endoderm	
Fetus	0.9049 (*<0.0001*)	0.9376 (*<0.0001*)	0.2364 (*0.2259*)	0.1310 (*0.5063*)	0.6578 (*0.0001*)
Amnion		0.9219 (*<0.0001*)	0.1790 (0.3621)	0.1091 (0.5805)	0.6290 (*0.0003*)
Yolk Sac mesoderm			0.2769 (0.1538)	0.0916 (0.6428)	0.6691 (*0.0001*)
Yolk Sac endoderm				0.4882 (*0.0084*)	0.3766 (*0.0482*)
Parietal endoderm					0.2280 (0.2432)

The % GPI-1A was considered to be significantly correlated between two tissues when $P < 0.05$ (shown in italics)



Graphs showing relationships in % GPI-1A between pairs
ues from 28 chimaeric conceptuses. The % GPI-1A in the
s positively correlated with that in the amnion (*A*), yolk
toderm (*B*) and with the placenta (*E*) but not correlated
he yolk sac endoderm (*C*) or parietal endoderm (*D*). The
I-1A in the two primitive endoderm derivatives (yolk sac
rm and parietal endoderm) is also positively correlated (*F*).
ble 4 for significance levels

1B) was also excluded from the amnion and yolk
nesoderm. The two primitive endoderm tissues
d different frequencies of loss of one cell popula-
Four of the parietal endoderm samples had only
A activity whereas all 28 yolk sac endoderm sam-
ad both GPI-1A and GPI-1B. (This assumes that
4% GPI-1B in the yolk sac endoderm of XE-11
enuine and not contamination from the yolk sac
derm layer which had 24.0% GPI-1B).

Discussion

For the fetus, amnion, yolk sac mesoderm and the parietal endoderm the distributions were slightly skewed in favour of a higher percentage of GPI-1A and when one cell population was excluded from these tissues it was always GPI-1B. In principle the AF_2 cells could be preferentially allocated to the inner cell mass (ICM) or have a selective advantage during subsequent development and growth of some tissues. (Preferentially allocation of AF_2 cells to the ICM might occur, for example, if embryos of this strain were usually developmentally more advanced than their BF_2 partners; see Spindle, 1982). In their studies of mouse chimaeras, Mullen and Whitten (1971) considered that a given strain combination was "developmentally balanced" if there was a large proportion of animals with a high degree of chimaerism (containing between 30% and 70% of each cell population). On this basis, the $AF_2 \leftrightarrow BF_2$ strain combination is probably slightly unbalanced (10/28 fetuses were within the 30–70% range; 18/28 were within 20–80%). No imbalance was apparent in the yolk sac endoderm or placenta and the mean % GPI-1A in these two tissues was significantly different from those in the other four tissues. If the imbalance is significant in the other tissues, many explanations are possible (e.g. preferential allocation of AF_2 cells to the inner cell mass followed by selection in favour of BF_2 cells in the yolk sac endoderm) but this cannot be resolved without analysis of chimaeras at earlier stages.

The results from the present series of $AF_2 \leftrightarrow BF_2$ chimaeras support the conclusions drawn from a previously studied, smaller group of C3H/HeH↔C3H/HeHa-*Pkg-1*^{a}/Ws chimaeras (West et al. 1984). The proportions of the two cell populations were found to be highly positively correlated within the primitive ectoderm lineage (between the fetus, amnion and yolk sac mesoderm) and within the primitive endoderm lineage (between the yolk sac endoderm and the parietal endoderm) but no correlation (either positive or negative) was seen between the two lineages. The chimaeras used in the present study

ere of a different strain combination (embryos from vo different F_2 stocks) from those in the previous study mbryos from partially congenic strains) and the proortions of the two cell populations were analysed with different enzyme marker (GPI-1 rather than PGK-1).), the complete agreement between the two studies, the relationships found between the tissues of the imitive ectoderm and primitive endoderm lineages, iggests that these observations have general validity.

Other similarities exist between the two studies. In oth studies some chimaeric conceptuses had non-chiaeric tissues, where one of the two cell populations ad been lost. Three consistent trends have emerged but e numbers of chimaeras involved are small and the ends do not reach statistical significance in either study sted by Fisher's Exact Test). First, each study has ore cases where one cell population is excluded from e fetus, amnion and yolk sac mesoderm rather than om one of these tissues alone (3/28 versus 0/28 in the esent study, $P=0.118$; 5/12 versus 1/12 in the previous udy, $P=0.078$). The second trend involves the primie endoderm lineage in chimaeric conceptuses. The oportion of non-chimaeric samples was higher for the rietal endoderm than the yolk sac endoderm in both udies (4/28 versus 0/28, $P=0.056$; 3/12 versus 1/12, $=0.295$) and the yolk sac endoderm retained both cell pulations more frequently than any other tissue. A ird trend to emerge from both studies was that one pulation was more commonly lost from the whole imitive ectoderm lineage than from the whole primie endoderm lineage (3/28 versus 0/28, $P=0.118$; 5/12 rsus 1/12, $P=0.078$). More data are required to prorly test the biological significance of these three trends.

Two significant observations, concerning the placenta chimaeric conceptuses, were made in the present udy. First, the proportion of GPI-1A in the placenta s positively correlated with that in each of the three imitive ectoderm derivatives. This was unexpected beuse, aside from the maternal part of the placenta, the acenta is mostly derived from the polar trophectoderm g. 6) with only minor contributions from the primitive doderm (where the parietal endoderm and yolk sac n the placenta) and primitive ectoderm lineages (umical cord, allantois and chorionic mesoderm). Experints with blastocysts that were reconstituted from getically distinct trophectoderms and inner cell masses Rossant and Croy (1985) to estimate that about 4% the 13–15 day mouse placenta was derived from the M. The ICM derivatives were confined to the inner rt of the placenta (labyrinthine trophoblast region) d included the fetal blood capillaries and mesenchyme s the endodermal sinuses.

The positive correlations between the placenta and three primitive ectoderm derivatives, in the chimaeric nceptuses, are likely to be genuine because the statistitest used (Spearman's correlation coefficient) is based ranking the data and so was unaffected by the calcuon used to correct for the proportion of maternal I-1A in the placenta. (The same results were obtained h uncorrected data.) There are two explanations for se positive correlations. First, there may be exchange

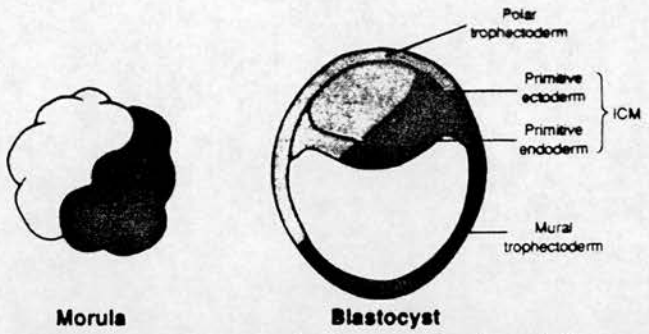


Fig. 6. Diagram showing the development of a chimaeric bastocyst (right) from a morula (redrawn after West et al. 1984). The diagram assumes that there is little or no cell mixing at this early stage. The cells derived from the two 8-cell embryos (*shaded black and grey*) occupy two hemispheres of the morula and these domains are retained in the blastocyst. If there was no cell mixing, each hemisphere would represent a single "coherent clone" of cells. In reality there will be more cell mixing than is implied by the diagram but it is unlikely to be extensive (Garner and McLaren 1974; Kelly 1979; Ziomek and Johnson 1982). See text for discussion

of cells between the polar trophectoderm and the underlying ICM cells that form the primitive ectoderm. This would support the hypothesis that, in the early blastocyst, some polar trophectoderm cells are derived from the ICM (Handyside 1978; Cruz and Pedersen 1985). Rossant and Croy (1985) also invoked this hypothesis to explain some anomalous conceptuses which had high ICM contributions in the placenta. Second, the positive correlation could be explained on a positional basis that does not imply a shared developmental lineage in normal (non-chimaeric) embryos. If cell mixing has not been extensive, patches of cells of the same population are likely to straddle the boundary between the polar trophectoderm and the underlying primitive ectoderm. We have no evidence that allows us to choose between these two possibilities but Fig. 6 may help to evaluate the feasibility of the second alternative.

Fig. 6 shows a possible distribution of two cell populations (shaded grey and black respectively) in a chimaeric blastocyst. Little or no cell mixing in assumed to occur immediately after aggregation (Garner and McLaren 1974; Kelly 1979; Ziomek and Johnson 1982), so that the black and grey cells are illustrated as each occupying a hemispherical domain. The allocation of ICM to primitive ectoderm and primitive endoderm may generate tissues with very different proportions (Falconer and Avery 1978; West et al. 1984) and in Fig. 6 the primitive ectoderm is shown as predominantly grey while the primitive endoderm is mainly black. Several factors, other than cell selection, may affect the proportion of placental trophoblast cells, derived from the trophectoderm layer of the blastocyst. It could be argued that if the two aggregated embryos occupy hemispherical domains and the trophectoderm cells are allocated on the basis of their outside position, the trophectoderm should be initially composed of approximately equal number of cells from each embryo. However, the proportion may deviate from the 50% illustrated in Fig. 6 for several reasons.

irst, one or both daughter cells of each 8-cell stage
omere may subsequently become trophectoderm
(Johnson and Maro 1986; Pickering et al. 1988)
ne embryo may produce more trophectoderm cells.
mechanisms that control this choice are imperfectly
rstood and may operate differently in chimaeric ag-
ates that involve twice as many cells. Second, early
ling blastomeres tend to contribute to the ICM more
iently than later dividing cells, so if developmental
chrony exists between the two aggregated embryos
may affect their contributions to the trophectoderm
ıdle 1982). Perhaps most significantly, most of the
ntal trophoblast cells are derived from a small
ber of polar trophectoderm cells. (The mural tro-
toderm cells stop division soon after losing contact
the inner cell mass, so it contributes only a few
inally differentiated trophoblast giant cells to the
day conceptus; Copp 1978; 1979.) In Fig. 6 the
ı of grey cells straddles the boundary between the
trophectoderm and the primitive ectoderm so that
lar trophectoderm composed entirely of grey cells
ies a primitive ectoderm that is predominantly but
xclusively grey.

ıe second significant observation, concerning the
nta, was that the distribution of % GPI-1A in the
nta was U-shaped and unlike that of any other
(Fig. 4); in 17/28 placenta samples the proportion
e minor component was 10% or less. It is unlikely
he procedure used to correct for the maternal con-
ıation generated the U-shaped distribution because
in et al. (1984) commented on a similar observa-
In this case 21/26 of the placentas of aggregation
aeras, made by aggregating normal diploid embryos
diploid embryos containing a Robertsonian translo-
ı, had a minor component that contributed 10%
ss. These two cases of U-shaped distributions of
d↔diploid chimaeric placentas suggests that the
trisomy-15↔diploid chimaeras that had unbal-
and widely differing placental contributions (0%,
90% and 100% respectively; Epstein et al. 1984)
ıot be as unusual as they first appear.

e U-shaped distribution suggests that the placental
activity is derived from a small number of cohe-
ones (either a small number of precursor cells or
ly a larger number of cells if the two cell popula-
are not intimately mixed so that the coherent clones
rge; see West 1978). In a chimaeric blastocyst with
ll populations that are not finely intermingled the
trophectoderm cells may often all be from the same
ıpulation (grey cells in Fig. 6) and so the placental
ıblast will be derived from only one cell popula-
minor contribution of placental tissue is derived
he other two primary developmental lineages (see
and may have very different proportions of the
PI-1 allozymes.

e the mural trophectoderm contributes few cells
ılacenta, it will contribute a negligible proportion
total GPI-1 activity at $12^1/_2$ days. Thus, if one
pulation of a chimaeric blastocyst was confined
mural trophectoderm, it may not be detectable
-1 analysis of the $12^1/_2$ day conceptus. The five non-chimaeric conceptuses shown in Table 2 (four AF_2 and one BF_2) could have arisen either as a consequence of technical failure or confinement of one cell population to the mural trophectoderm lineage.

In conclusion, we have shown that, although as a population the placentas of chimaeric conceptuses are balanced with respect to the % GPI-1A (mean close to 50%), individually most placentas are extremely unbalanced in their chimaeric composition (<10% or >90% GPI-1A). This non-random composition of the chimaeric placentas is in contrast to the widely held assumption that there is normally a random distribution of embryonic cells in chimaeras. A more detailed understanding of the cellular distributions in control aggregation chimaeras should provide a better basis for use of chimaeras to evaluate the developmental potential of cells, with various types of developmental defects. A more direct analysis of the distribution of two cell populations in chimaeric blastocysts and egg cylinder stage embryos, using an in situ marker (e.g. Thomson and Solter 1988, 1989) may also provide useful information about cell allocation, although it may be necessary to reduce the cell numbers to make chimaeras a more realistic model of the normal embryo.

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